AD_____

Award Number: W81XWH-07-1-0181

TITLE: Modulation of Stem Cells Differentiation and Myostatin as an approach to Counteract fibrosis in Muscle Dystrophy and Regeneration after Injury

PRINCIPAL INVESTIGATOR: Nestor F. Gonzalez-Cadavid, Ph.D.

CONTRACTING ORGANIZATION: Charles Drew University
Los Angeles, CA 90059

REPORT DATE: March 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-03-2008	Annual	01 MAR 2007 - 28 FEB 2008

4. TITLE AND SUBTITLE

Modulation of Stem Cells Differentiation and Myostatin as an approach to Counteract fibrosis in Muscle Dystrophy and Regeneration after Injury

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0181

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Nestor F. Gonzalez-Cadavid, Ph.D.

E-Mail: ncadavid@ucla.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Charles Drew University
Los Angeles, CA 90059

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Muscle derived stem cells (MDSC) were isolated from the myostatin wild type mouse (Wt), the myostatin knock out mouse (Mst ko), and a DMD mouse model (mdx), and shown that: 1) Implants of Wt MDSC regenerate in vivo smooth muscle tissue and other cell types in injured or impaired tissue and correct the functional dysfunction created by the loss of these cells and myofibroblast generation, and also convert into myofibers in skeletal muscle, thus showing MDSC responsiveness to in vivo paracrine modulation of cell lineage. 2) Wt MDSC express an embryonic stem cell marker, Oct-4, and cells positive for this marker were located in vivo in the skeletal muscle using a transgenic mouse model that detects Oct-4 expression with a reporter gene; 3) Wt and Mst ko MDSC were unexpectedly resistant to in vitro paracrine and autocrine modulation of myogenesis by effectors of the myostatin pathway, but our previous results suggest that MDSC differentiation is responsive in vivo to factors in the tissue environment. 4) Myostatin pro-fibrotic role was shown in a multipotent cell line, the C3H 10T1/2, and in vivo in stem cells in connective tissue, thus confirming our proposal to counteract myostatin in MDSC or in the host tissue for the therapy of DMD

15. SUBJECT TERMS
Myostatin, muscle dystrophy, stem cells, myogenesis, oct-4

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	102	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

Introduction

The **overall objective** of this whole grant is:

To investigate in the mdx mouse a novel therapeutic approach for Duchenne's muscular dystrophy **(DMD)** based on the inhibition of myostatin **(Mst)** expression and/or activity, for the alleviation of fibrotic and fatty degeneration of the muscle, that would also facilitate the differentiation of transplanted dystrophin+ **(D+)** muscle-derived stem cells **(MDSC)**, in order to ameliorate disease progression.

This will be achieved by: a) comparing the in vitro myogenic and fibrogenic/adipogenic potential of MDSC from D-/Mst+, D+/Mst+ or D+/Mst- mice; b) blocking myostatin expression by gene transfer of myostatin short hairpin RNA (Mst shRNA), or transplantation of D+ MDSC engineered with Mst shRNA, and measuring the myogenic/fibroadipogenic balance, dystrophin expression, and muscle function; and c) combining this with the inhibition of myostatin activity by follistatin.

The **specific objective 1** to be fulfilled in **Year 1** was: To determine in vitro whether the inhibition of myostatin in MDSC from dystrophic and normal gastrocnemius: a) improves the balance between myogenic and fibro-adipogenic differentiation; b) occurs via Smad pathways and involves paracrine interactions

The predicted Milestones for Year 1 are: A) In vitro comparison of myogenic, fibrogenic and adipogenic potential of stem cells from intact and regenerating muscle from mdx, wt and Mst(-/-) mice; b) effects on these differentiation potentials produced by blocking myostatin and its Smad pathway

Description of research accomplishments

In our time table of work, we had projected that during Year 1 we would carry out Tasks 1 and 2 within Objective 1**.**

Task 1. To assess the influence of dystrophin or myostatin deficiency on the myogenic and fibro-adipogenic potential of stem cell-enriched cultures from gastrocnemius

The gastrocnemius will be excised from 3 month old mdx mice, and from Mst(+/+) and Mst(-/-) mice(n=8/group, total 24 mice). MDSC will be isolated (pP6 fraction), followed by Sca1+ selection, and tested for myogenic, fibrotic (myofibroblast), and adipogenic differentiation, in monocultures, and in dual cultures for paracrine and yuxtacrine interactions with C2C12 myoblasts.

Cell markers throughout this proposal will be compared by immunocytochemistry (ICC) or dual fluorescence, combined with quantitative image analysis (QIA), and/or quantitative western blot and/or real time RT/PCR.

Outcomes: A) Cell type characterization: a) yield and replication rate; b) stem cells (Sca1, CD34) and satellite cells (Pax7); B) TGFβ family expression: myostatin and TGFβ1; C) Lineage commitment: a) myogenesis, by MyoD (early), myogenin (intermediate), and MHC II (late); b) fibrogenesis by alpha smooth muscle actin (ASMA) and collagen I/III ratio; c) adipogenesis by Oil-Red-O count, PPARγ, and C/EBPα

Results obtained so far

References identified as **G-1** to **G-9** pertain to our group papers and abstracts related and acknowledging this grant, as given under Reportable outcomes. All other references are listed within the bibliography of those papers, or a few identified with numbers in the text and listed under References. This includes other papers from our groups not directly related to our grant.

a. MDSC are able to differentiate into different cell lineages

We have obtained, by applying the pre-plate procedure to gastrocnemius tissue **(Fig. 1),** the cell fractions derived from various muscle cell types which are identified as pP1 through pP6, from: a) wild type mouse **(Wt);** b) myostatin knock-out mouse **(Mst-ko)**; c) and mdx mouse **(mdx).** So far, the pP6 cell population containing the MDSC, which is the only one planned to be employed in this grant, has been characterized in the experiments described for Tasks 1 and 2, for Wt and Mst ko. Briefly:

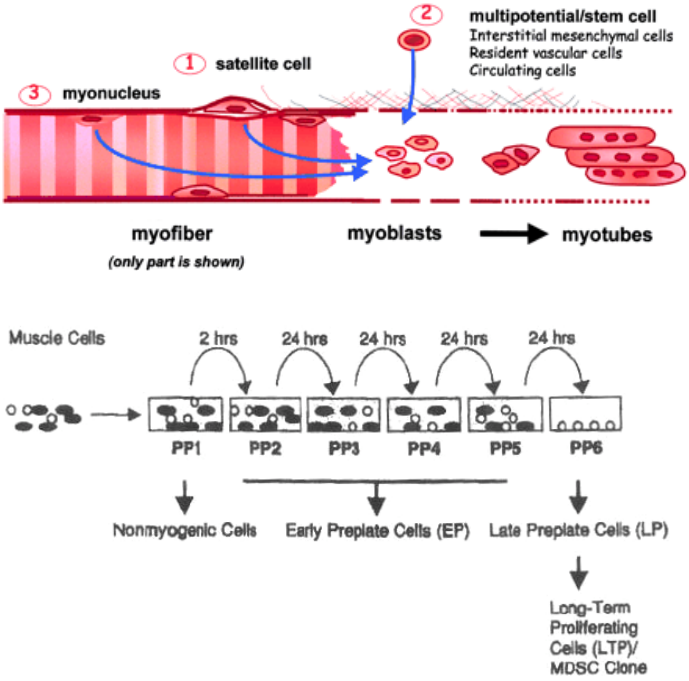


Fig. 1. Schematic representation of the putative location of MDSC in the skeletal muscle, and of the pre-plate procedure used for their isolation.

We have shown **(G-1,G-8)** that Wt MDSC incubated in vitro under the different media described in the grant are able to generate not just myotubes and myofibroblasts, the main topic of this grant, but osteoblasts and smooth muscle cells, as shown on **Fig. 2**. The ability to form myofibroblasts and smooth muscle cells was corroborated by western blot analysis where α-smooth muscle actin **(ASMA),** a marker that is shared by both types of cells, was clearly expressed under low conditions of serum, and the band for calponin, a specific marker for smooth muscle cells was also visible (not shown).

It was also found in vivo, by injecting DAPI-labeled MDSC into a tissue rich in smooth muscle, such as the penile corpora cavernosa of aged rats, that these cells were able to generate smooth muscle cells as recognized by ASMA immuno-fluorescence for ASMA **(Fig. 3**). Elucidation of this and other co-localizations will be performed in the current proposal using the UCLA fluorescent confocal microscope that allows to optically go down to 0.5 um or less optical sections to establish with reasonable certainty the overlapping of the implanted nuclei with the endogenous cells or fibers. This confirms the differentiation of the implanted stem cells into smooth muscle cells lining up the cisternae of the corpora (magenta in the overlay).

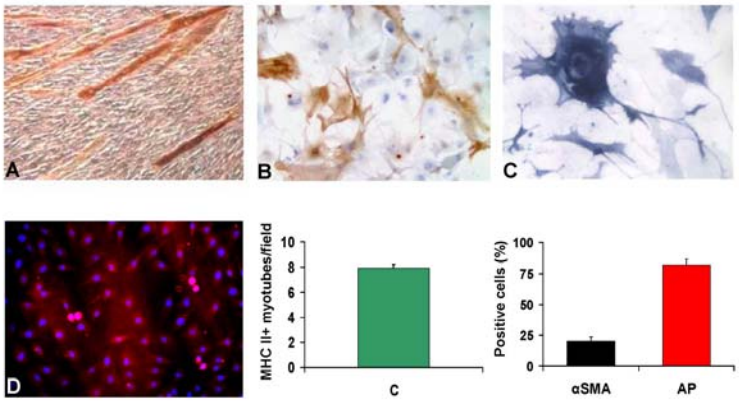


Fig. 2. Wt MDSC are able to originate different cell lineages when incubated in vitro with different media. Incubations were carried out for 2-3 weeks and cells were subjected to immunocyto-chemical or cytochemical staining. A: MHC-II for skeletal myofibers; B: ASMA for myofibroblasts or SMC; C: alkaline phosphatase (AP) for osteoblasts; D: immunofluorescent red staining for calponin super-imposed to general nuclear staining with DAPI; Bar grphs: QIA for MHC-II, ASMA, and AP

Other procedures were also employed to demonstrate in vivo the MDSC differentiation into smooth muscle cells. This MDSC implants restored the normal erectile function induced by the corporal smooth muscle in the animal by counteracting the loss of corporal smooth muscle cells induced by aging (not shown).

Since we intend to apply some of the MDSC into the skeletal muscle by using scaffolds that may facilitate retention in the site of injection and migration into the diaphragm of the mdx mouse, we grew these cells onto two types of scaffolds: pig small intestinal submucosa **(SIS)** (cells stained with hematoxylin and visualized on inverted light microscope) and polymeric scaffolds (DAPI-stained MDSC visualized with regular fluorescent microscope) **(G-7).** These cells attached to the scaffolds can convert into smooth muscle cells (ASMA+) or skeletal myofibers (MHC-II+) according to whether they are incubated with regular or myogenic medium**.**

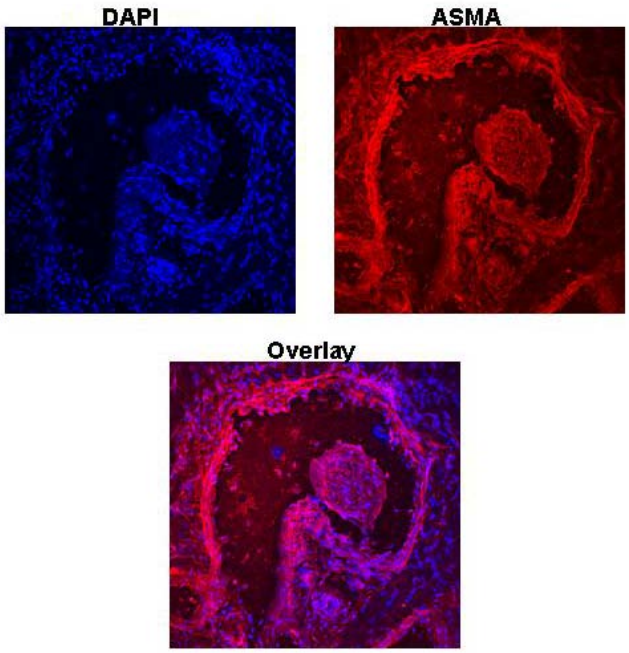


Figure 3. The stem cell nature of Wt MDSC and response to paracrine effects is evidenced by their conversion into SMC when implanted into the rat corpora cavernosa and this can be ascertained more precisely by confocal microscopy in 0.5 um sections. Top left: blue filter for DAPI-stained nuclei; Top right: cells immunostained for ASMA; Bottom: merge of both fields. Amplification: 200X.

Moreover, we have shown that SIS implants with DAPI-labeled MDSC can regenerate urogenital tissue, in this case vagina in female rats that had been both hysterectomized and ovariectomized, and then implanted at the top of the vagina with the scaffold (not shown). These scaffolds are going to be tested in this proposal.

MDSC also proved to be effective in converting themselves into cardiomyocytes in vitro, as demonstrated by immunocytochemistry for troponin, GATA 4, and other cardiac markers and by western blot (not shown). Finally, DAPI-labeled MDSC injected into the tibialis skeletal muscle were visible around the myofibers in sections stained for MHC-II with a Texas red streptavidin/biotin system, both in cross sections and in longitudinal sections (not shown).

Altogether, this confirms the stem cell nature of MDSC from the Wt. The pP6 from the Mst ko also are able to generate osteoblasts and myofibroblasts, however, surprisingly they did not in vitro generate myotubes (see next section).

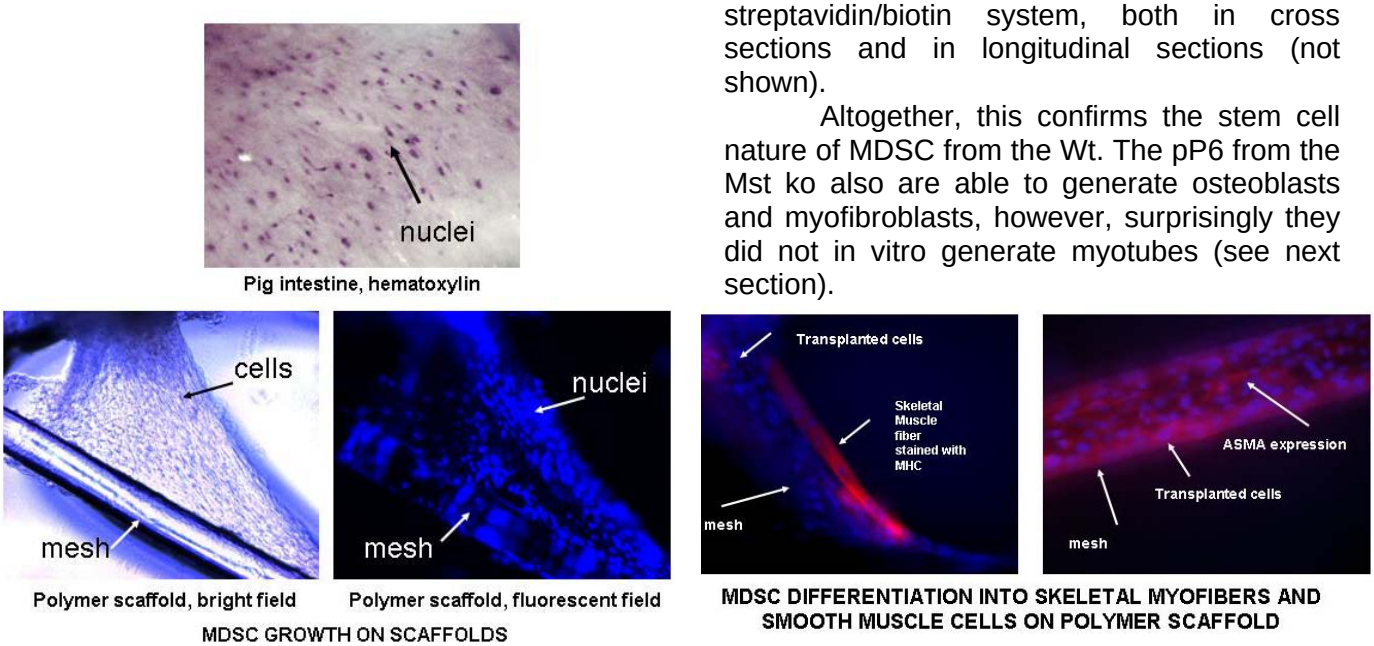


Fig. 4. Wt MDSC grow in vitro on scaffolds and differentiate into the desired cell lineages. Pig intestine: small intestinal submucosa (SIS)

The same paradigms described above will be applied to the Mst ko and mdx pP6 cell fractions

containing MDSC. In the case of the mdx, these fractions have just been isolated and tests are in progress.

b. Expression of stem cell markers in MDSC

It is known that MDSC express both Sca1 and CD34, and as reported as preliminary results for this grant our Wt and Mst ko MDSC express these markers. We have recently been able to detect similar CD34+ cells in tissues respectively rich in fibroblast or smooth muscle in the rat penis using either immunohisto-chemistry or immunofluorescence**,** and similar results were also obtained for Sca1 **(G-1,G-8)** (not shown), thus suggesting that endogenous stem cells similar to MDSC, albeit most likely of different origin, are also present in these tissues. However, our concerns for this grant are: a) how to identify MDSC in vitro with more specific markers; and b) how to locate MDSC in skeletal muscle tissue sections that would differentiate myofibers from interstitial tissue.

DNA MICROARRAY IN RNA EXPRESSION POSSIBLE MDSC MARKERS

OMM	No.	Abbrev.	Full Name	A	B
405	199	Myst 4	Myst histone acetyltransferase	45	34
	200	Nanog	Nanog homeobox	30	29
	209	Notch 3	Notch3 gene homolog 3	53	56
	222	Pou 5 fi (Oct 4)	Pou domain, class 5	23	8
	282	Wnt 1	Wingless-related MMTV integration site 1	20	72
055	29	CD63	CD63 antigen	259	ND
037	20	Ckm	Muscle creatin kinase	ND	32
	73	Mb	Myoglobin	ND	112

OMM: Array number; Relative intensities: A: 2.5% fetal bovine serum; B: 20% fetal bovine serum; ND: Not Determined

Fig. 5. Detection of Oct-4 mRNA expression in Wt MDSC, among other potential stem cell markers. Cells were grown under low or high serum for 3 weeks

By applying DNA microarrays on RNA isolated from Wt MDSC growing under low serum concentration (favoring cell differentiation) and high serum concentration (favoring proliferation) we could identify a series of potential stem cell markers **(Fig. 5),** and among them Oct-4, also known as Pou5fi, the most representative of embryonic stem cell markers, which has also been shown in tumor cells (cancer is supposed to be a stem cell disregulation) and in adult tissues where it is considered to represent stem cells present in those locations. Oct 4 is in addition the gene that recently was employed to transform normal adult differentiated cells into embryonic-like stem cells **(1,2).** In addition, we have identified a strong Oct-4 mRNA expression in the gastrocnemius, and obviously in the testis where germinal stem cells are present, by RT/PCR. Surprisingly, the expression in the MDSC was very faint but still detectable and in the MDSC **(Fig. 6)**

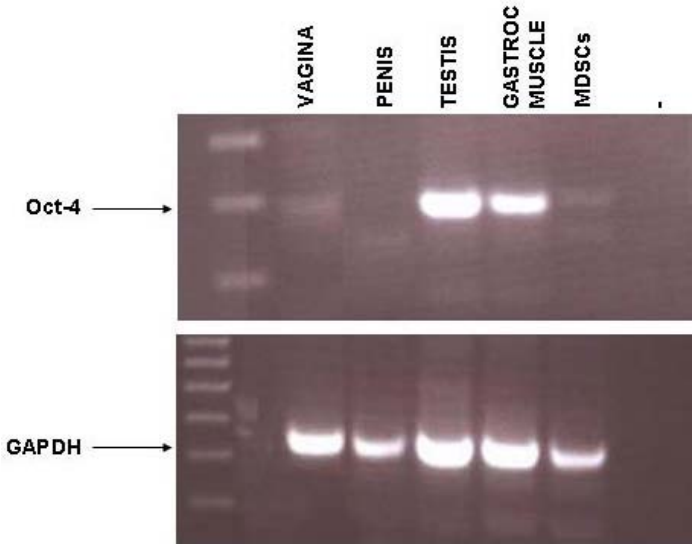


Fig. 6. The embryonic stem cell marker Oct-4 is detectable in Wt MDSC and skeletal muscle mRNA by RT/PCR

The availability at our institution (Dr. H-Y Lue), of a transgenic mouse expressing the reporter gene Egfp under the Oct-4 promoter **(3-5**) opened up the possibility of employing this animal to visualize in the penis the cells that by expressing gfp and hence be green fluorescent, would be potential endogenous stem cells.

Fig. 7 top left shows that the positive control, e.g., the testis, has a series of green fluorescent nuclei around the seminiferous tubules, that may correspond to the germ cells or testis stem cells.

The wild type (same genetic background) mouse testis virtually does not exhibit auto-fluorescence (not shown). Nuclear staining by DAPI anti-fade indicates that only a fraction of the nuclei overlap with green fluorescence (see merge). The same green fluorescent nuclei, but only around certain myofibers in the tissue are found in cross-sections of the gastrocnemius, but only with pale fluorescence **(top right).** Other myofibers show strong fluorescence spread throughout the figure, which could mean a recent Oct-4+ cell fusion or be an artifact **(bottom left).** The wild type mouse skeletal muscle does not have self-fluorescence other than collagen fibers, that is eliminated by a similar exposure time and background correction in both the Oct-4 gfp and wild type tissues. Sagital sections show merges of green Oct-4+ nuclei with red propidium iodide stained total nuclei, in yellowish green. These observations are being confirmed with additional sections and with dual staining for MHC-II.

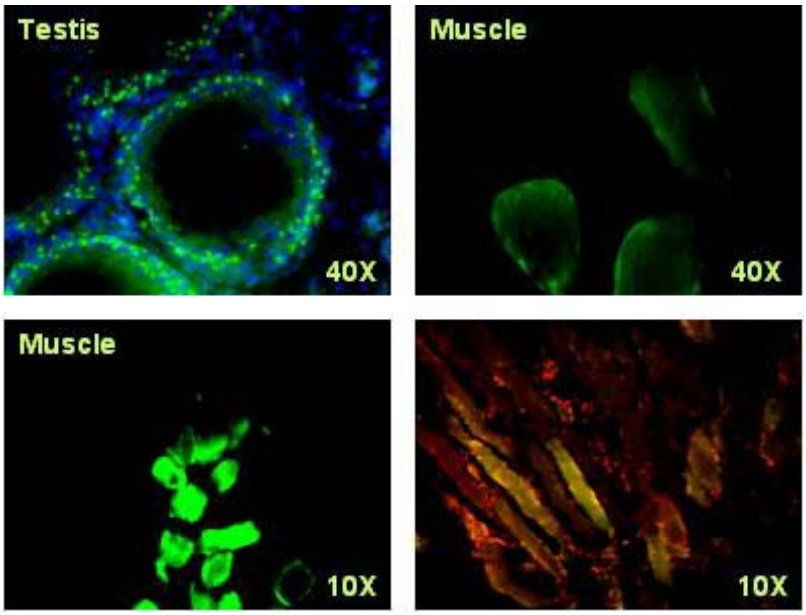


Fig. 7. Detection of potential stem cells in the skeletal muscle of the Oct-4-gfp mouse by green fluorescence, using the testis seminiferous tubules as positive control. Frozen sections of testis and gastrocnemius were examined where nuclei were visualized with DAPI (blue) (top left) or propidium iodide (red) (bottom right),. The respective images were merged in both cases. The background fluorescence was corrected by adjusting with wild type tissue sections.

Task 2. To assess the influence of blocking myostatin activity and/or expression on the myogenic and fibro-adipogenic potential of stem cell-enriched cultures from dystrophic and wild type gastrocnemius, and whether this process is affected by the Smad pathway

MDSC from the 1) mdx, and 2) Mst(+/+) groups (Task 1), will be subjected to anti-myostatin treatments and tested for differentiation as in Task 1: a) anti-myostatin antibody; b) follistatin; c) transfection with AdV-Mst shRNA. Controls will be MDSC from the 3) Mst(-/-) group, with either: d) recombinant myostatin (Mst-110); or b) transfection with AdV cDNA for myostatin (AdV-Mst cDNA), with and without plasmid Smad7 cDNA, and tested as in Task 1.

Outcomes: A) TGFβ family expression: myostatin and TGFβ1; B) Lineage commitment: a) myogenesis by MHC II; b) fibrogenesis by ASMA; c) adipogenesis by Oil-Red-O count.; C) Smad pathway: Smad members and their phosphoproteins.

Results obtained so far

a. Effects of recombinant myostatin and testosterone

We have carried out all the cell incubations programmed for this task for the Wt and Mst ko MDSC. However, in the case of mdx MDSC, because of difficulties in amplifying these cell cultures we could only recently investigate their myogenic potential and experiments are ongoing. We found out that Wt and Mstko MDSC do not undergo any true adipogenic differentiation even in the adipogenic medium that was very effective for our previous studies on testosterone and myostatin effects on the pluripotent cell line C3H 10T1/2 **(6,7),** despite the fact that this is the most usual conversion undergone by stem cells. This may be beneficial, since the mdx skeletal muscle undergoes lipo-

degeneration, and an additional adipogenic differentiation of the implanted MDSC would exacerbate the dystrophy.

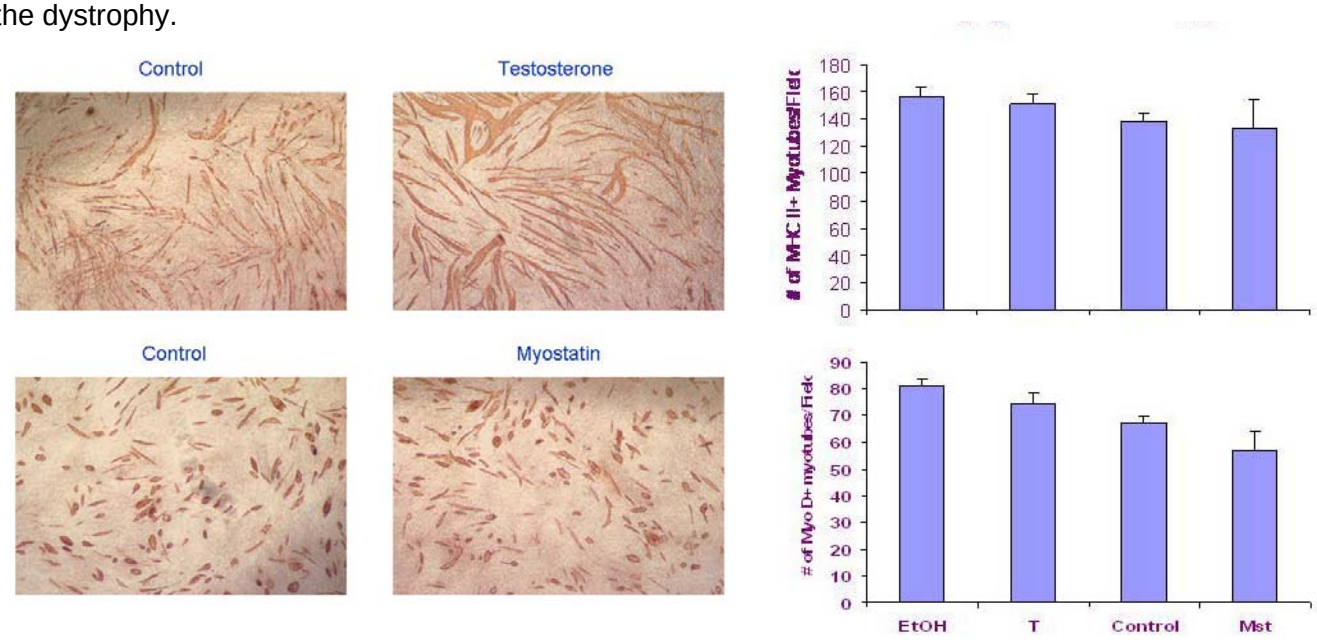


Fig. 8. Lack of effects of recombinant myostatin or testosterone on the in vitro myogenic differentiation of Wt MDSC. Right panels: MDSC were switched to myogenic medium upon confluence and incubated for 3 weeks with either agent. Cells were immunostained for MHC-II. Left bar graphs: quantitative image analysis of myotube number. Eth: 0.05% ethanol; testosterone contains same amount of ethanol. The others do not have ethanol

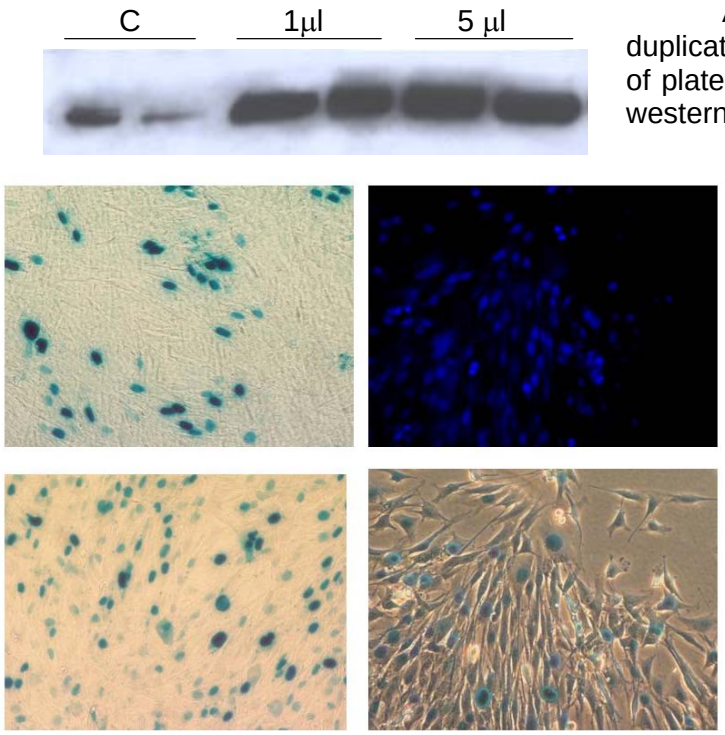


Fig. 9. Transduction of Wt MDSC with adenoviral preparations of myostatin cDNA or shRNA. Top: western blot of HEK cells transduced with AdV Mst cDNA; 50 kDa band shown. Middle four panels: a) upper left: X-gal staining of MDSC infected in 6 well plate with $2x10^7$ ivu of AdV-mst-shRNA (200X); b) Upper right: DAPI-stained MDSC infected with $1.2x10^8$ ivu of Adv-mst-shRNA (200X). c) Lower left: Same field as b), but X-gal stain under bright field; d) Lower right: Same field and staining as c) but phase contrast to shown cell borders.

All myogenic incubations were carried out in duplicate on 6-well collagen coated plates, one set of plates for immunocytochemistry and the other for western blots, unless stated, allowing cells to reach confluence and then switching to Hedrick's myogenic medium **(8).** Wt MDSC formed in this medium very distinctive myotubes after 2-3 weeks incubation, although passage number seemed to affect considerably the size and number of WT nuclei per myotube. **Fig. 8** shows that myotubes from control MDSC (containing 0.05% ethanol only, to mimic the testosterone wells) from passage 25 **(left top)** were bigger and more branched than the ones from passage 45 **(left bottom).** According to Huard's group MDSC continue actively replicating, an observation that we agree with, but they did not report changes in myogenic ability **(9,10)**. However, addition of recombinant myostatin (4 ug/ml) that had considerably reduced the number of myotubes in C3H 10T1/2 multipotent cells **(8)** was unable to affect significantly the myogenic differentiation of MDSC. A similar lack of response of MDSC differentiation

occurred with 100 nM testosterone, that was used as positive control, since it stimulates myogenesis in multipotent C3H 10T(1/2) cells **(7).**

Significantly, we found that testosterone virtually doubled the size of myotubes as determined by quantitative image analysis expressing as total MHC-II+ area divided by the number of myotubes, e.g., it exerted myotube hypertrophy. This was probably due to the stimulation of myoblast fusion rather than of MDSC differentiation, and in the case of myostatin we are still analyzing the data to determine whether it exerts any significant effect, namely size reduction. A similar lack of response to the expression of MyoD as early myogenic marker by either testosterone or myostatin was observed by immuno-cytochemistry (not shown). This was confirmed by quantitative western blot analysis for MHC-II and MyoD, where no significant effects on MDSC myogenic differentiation were detected (not shown). MDSC did not express MHC-I.

A very important and still unexplained observation is that the Mst ko MDSC were not able to originate myotubes or expression of MHC-II, even after 4 weeks of incubation in myogenic medium in at least five different experiments by four different operators and using passages from 20 through 30. Notably, there was a faint uniform expression of the early marker MyoD in mononucleated MDSC as evidenced by immuno-cytochemistry and western blot (not shown). We don't know the reason for this failure to form myotubes, when in fact the expectation is that the absence of mystatin should intensify myogenesis. This is an important aspect to elucidate with earlier passages and/or paracrine or juxtacrine effects

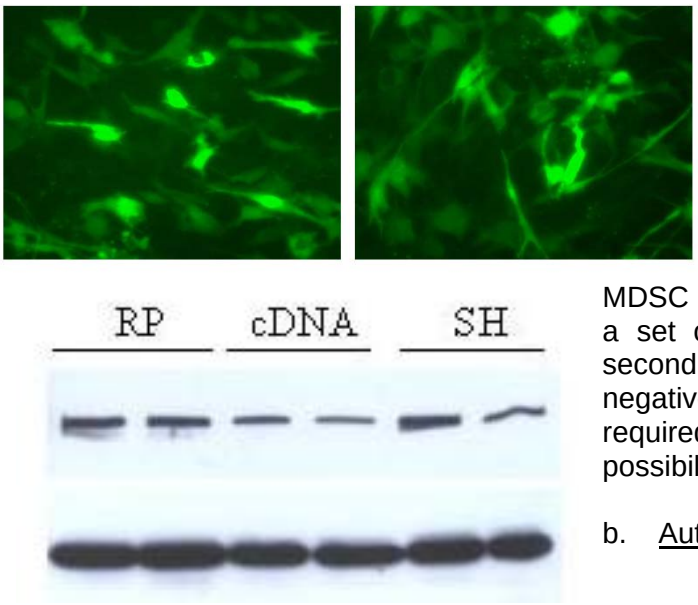


Fig. 10. Transduction of Wt MDSC with plasmid constructs of myostatin cDNA or shRNA. Upper panels: Left: approximately 10^6 MDSC were nucleofected with 1 ug of pMaxGFP (Amaxa), grown for two days, and visualized for green fluorescence (200X). Nucleofector program P24 using the Primary Smooth Muscle Cell solution. Right: Same conditions as left, except using program U25. Bottom: western blot for MHC-II (top) and GAPDH (bottom) for MDSC transfected with; RP: random primer; cDNA: AdV Mst cDNA; SH: AdV Mst shRNA

Another possible explanation is that in the serial pre-plating, the Mst ko pP6 fraction ended up as not being equivalent to the WT pP6. For this reason, we are currently testing myogenesis by pP2, pP3, pP4 and pP5 from both WT and Mst ko, to check on whether MDSC may be found in pP5 or pP4, This would require a set of multiple lineage differentiation assays. The second possibility is that myostatin, despite being a negative regulator of skeletal muscle, is however required for myotube formation. This is an intriguing possibility that needs careful examination.

b. Autocrine effects of myostatin

We have generated the plasmid and AdV constructs expressing Mst cDNA and its respective Mst sh RNA, that have been extensively tested in vitro by us **(G-2, 7).** The AdV MstcDNA is able to express the 50 kDa full length myostatin in HEK cells as shown by western blot **(Fig. 9 top)**, and has been successfully used in vivo to express myostatin in a connective tissue of the penis and induce fibrosis, thus indirectly confirming our hypothesis in the skeletal muscle **(G-3).** In turn, the AdV shMst RNA blocked myostatin expression in vitro and in vivo in the skeletal muscle **(11).** Since this latter construct expresses a reporter gene (β-galactosidase) in tandem with the Mst shRNA, we have used it for this grant at high titer to demonstrate that it is possible to get over 90% efficiency in the transduction of MDSC **(Fig. 9 micrographies).** This was shown by X-gal staining contrasting with total nuclei staining with DAPI.

We also tried the respective plasmid preparations, that include an additional construct: the randomer control, generated and previously tested by us **(G-2, 11).** In order to increase the efficiency

of transfection of these plasmids into MDSC we applied Nucleofection. This technique has proved to be a useful tool for moving DNA into many different cell types, but has not previously been used in MDSC (Amaxa, personal communication). We therefore tested two types of nucleofection medium (embryonic stem cell medium and basic smooth muscle cell medium) supplied by the manufacturer under nine different nucleofection programs, using the pMaxGFP (Amaxa) expressing gfp as a reporter protein We found that two programs (P24 and U25) in the presence of smooth muscle medium resulted in at least 60% Green Fluorescent Protein positive cells **(Fig. 10 top).**

However, despite these adequate transfection procedures, neither the plasmid or AdV preparations Mst sh RNA nor the Mst cDNA exerted any considerable effect on visual myotube formation by WT MDSC incubated for 2 weeks, or on MHC-II expression by western blot **(Fig. 10 bottom).** Moreover, the Mst Ko MDSC were not affected either. To rule out that the expression of myostatin decays with time in 90% transfected MDSC we are conductiong time courses of myostatin expression, correlating with X-gal staining in adjacent wells.

c. Effects of follistatin and antibodies against myostatin

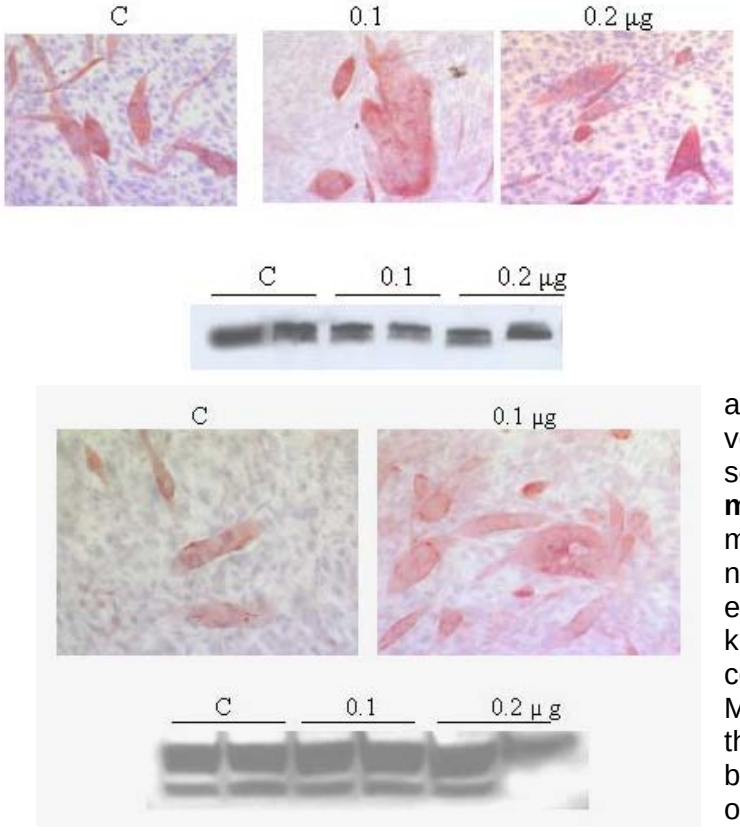


Fig. 11. Follistatin effects on myogenesis by Wt MDSC. Cells were incubated for 3 weeks as indicated and immunostained for MHC-II (top) or MyoD (bottom). In adjacent wells, extracts were obtained and assayed by western blots.

Another of the scheduled experiments was the incubation with follistatin, a protein that binds and inactivates myostatin, and therefore only WT MDSC were tested. Our expectations were that myogenesis would be stimulated. We did obtain some encouraging results, since follistatin acted as predicted, namely induced an increase in the number of MHC-II + myotubes assayed by immunocytochemistry from 285 per well to 413 at 0.1 ug/ml and 445 at 0.2 ug/ml. In addition, the myotubes had a very high content of nuclei, and displayed some bizarre forms **(Fig. 11 top micrographies)**, although we have not yet measured the mean size. However, this did not translate into an increase of MHC-II expression, as assessed by western blot (210 kDa band), so the observation needs confirmation. A similar situation occurred with MyoD+ myotubes that increased from 31 in the control to 59 in the follistatin (01 ug/ml), but without correspondence in the intensities of the 38 kDa MyoD band assessed by western blot.

The incubation of Wt MDSC with our monoclonal and polyclonal antibodies against myostatin developed previously **(see G-2,G-3,7,11)** was accompanied by substantial myotube formation, but the control MDSC monolayers were dettached (an unfortunately usual and unpredictable occurrence) and therefore the experiment must be repeated.

d. Effects of TGFβ1 and paracrine and juxtacrine effects of myoblasts

We also performed the scheduled experiments with TGFβ1, because of it being a member of the TGFβ superfamily that includes myostatin, and also because of the well known interaction of both proteins through the Smad pathway and their apparent mutual regulation of expression **(G-2,G-3),** and with myostatin antibodies that supposedly would counteract myostatin activity but not expression. In the case of TGFβ1, we observed at 5 ng/ml, but not at 2.5 ng/ml an intensification of myotube formation in the case of WT MDSC. This, although was not accompanied by stimulation of MyoD expression by immunocytochemistry, could be detected by western blot. There was no change in ASMA expression, and indicator of myofiboblast formation, and hence of fibrogenic differentiation. This is important since under conditions adequate for skeletal myogenesis there seems to be no formation of myofibroblasts.

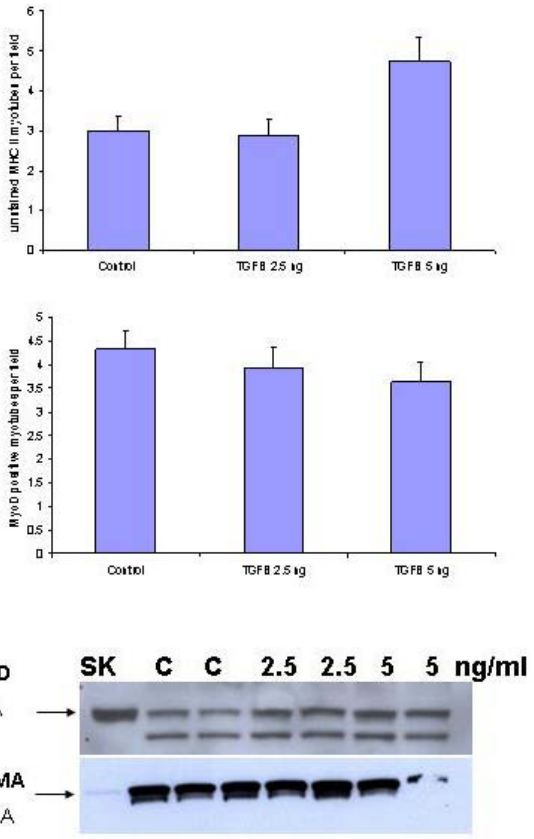


Fig. 11. TGFβ1 effects on myogenesis by Wt MDSC Cells were incubated for 3 weeks as indicated and immunostained for MHC-II or MyoD , determining the myotube number by QIA. In adjacent wells, extracts were obtained and assayed by western blots for MHC-II (not shown), MyoD and ASMA.

Wt and Mstko MDSC were incubated in myogenic medium with C2C12 cells, both in dual chambers where the C2C12 cells go on top of the insert and the MDSC remain at the well bottom, and in mixes of DAPI–labeled MDSC with C2C12 myoblasts at several ratios. These approaches measure paracrine and juxtacrine interactions, respectively.

The myogenic experiments with the mdx pP6 cells, and the ones with the pP2-5 from Wt and Mst ko are also ongoing. The Smad 7 experiment to inhibit the Smad pathway is pending.

Summary of papers (see appendix for full papers)

Paper #G-1. Nolazco G, Kovanecz I, Vernet D, Gelfand RA, Tsao J, Ferrini MG, Magee T, Rajfer J, Gonzalez-Cadavid NF. Effect of muscle-derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. BJU Int. 2008 Feb 21; [Epub ahead of print]

Objectives. Aging-related erectile dysfunction is primarily due to a loss of smooth muscle cells (SMC) in the corpora cavernosa. Current pharmacological treatments for erectile dysfunction simply induce an erection on demand, rather than alter the underlying aging related changes within the corporal tissue. In an attempt to replenish such a decreased SMC population, we investigated whether skeletal muscle derived stem cells (MDSC) convert into SMC both in vitro and in vivo, and in so doing ameliorate the erectile dysfunction of aged rats, as well as searched for endogenous stem cells in the rat corpora cavernosa.

Methods and Results. MDSC were obtained from mouse muscle, and shown by immuno cytochemistry for α-smooth muscle actin (ASMA) to originate myofibroblasts and SMC, discriminating SMC by calponin 1 expression. MDSC were labeled with DAPI and implanted for 2 and 4 weeks into the corpora cavernosa of young (5 month old) and old (20 month old) rats. The exogenous cells replicated and converted into SMC, as shown in corporal tissue sections by confocal immunofluorescence for proliferating cell nuclear antigen (PCNA), ASMA, and smoothelin, and also

by western blot for ASMA and PCNA. MDSC differentiation was confirmed by the activation of the ASMA promoter linked to β-galactosidase in transfected cells, both in vitro and implanted in the corpora. Putative endogenous stem cells were shown in corporal tissue sections and western blots by detecting CD34 and a possible Sca1 variant. Electrical field stimulation of the cavernosal nerve showed that implanted MDSC raised in aged rats the maximal intracavernosal pressure/mean arterial pressure levels above (2 weeks) or up to (4 weeks) those of adult rats.

Conclusions. MDSC implanted into the corpora cavernosa of aged rats converted into SMC and corrected erectile dysfunction, and endogenous cells expressing stem cell markers were also found in the untreated tissue. This suggests that exogenous stem cell implantation and/or endogenous stem cell modulation could be viable therapeutic approaches for aging-related erectile dysfunction

Paper #G-2 Artaza JN, Singh R, Ferrini MG, Braga M, Tsao J, Gonzalez-Cadavid NF. Myostatin promotes a fibrotic phenotypic switch in multipotent C3H 10T1/2 cells without affecting their differentiation into myofibroblasts. J Endocrinol. 2008 Feb;196(2):235-49.

Tissue fibrosis, the excessive deposition of collagen/extracellular matrix combined with the reduction of the cell compartment, defines fibroproliferative diseases, a major cause of death and a public health burden. Key cellular processes in fibrosis include the generation of myofibroblasts from progenitor cells, and the activation or switch of already differentiated cells to a fibrotic synthetic phenotype. Myostatin, a negative regulator of skeletal muscle mass, is postulated to be involved in muscle fibrosis. We have examined whether myostatin affects the differentiation of a multipotent mesenchymal mouse cell line into myofibroblasts, and/or modulates the fibrotic phenotype and Smad expression of the cell population. In addition, we investigated the role of follistatin in this process. Incubation of cells with recombinant myostatin protein did not affect the proportion of myofibroblasts in the culture, but significantly upregulated the expression of fibrotic markers such as collagen and the key profibrotic factors transforming growth factor-beta1 (TGF-beta1) and plasminogen activator inhibitor (PAI-1), as well as Smad3 and 4, and the pSmad2/3. An antifibrotic process evidenced by the upregulation of follistatin, Smad7, and matrix metalloproteinase 8 accompanied these changes. Follistatin inhibited TGF beta1 induction by myostatin. Transfection with a cDNA expressing myostatin upregulated PAI-1, whereas an shRNA against myostatin blocked this effect. In conclusion, myostatin induced a fibrotic phenotype without significantly affecting differentiation into myofibroblasts. The concurrent endogenous antifibrotic reaction confirms the view that phenotypic switches in multipotent and differentiated cells may affect the progress or reversion of fibrosis, and that myostatin pharmacological inactivation may be a novel therapeutic target against fibrosis.

Paper #G-3. Cantini LP, Ferrini MG, Vernet D, Magee TR, Qian A, Gelfand RA, Rajfer J, Gonzalez-Cadavid NF (2008) Pro-fibrotic role of myostatin in Peyronie's disease. J Sex Medic, in press

Main outcome measure. Myostatin expression in the human TA, the PD plaque, and their respective cell cultures, and myostatin effects on the PD-like plaque in the rat

Methods. Human TA and PD tissue sections, and cell cultures from both tissues incubated with myostatin and TGFβ1, were subjected to immunocytochemistry for myostatin and α-smooth muscle actin (ASMA). Cell homogenates were assayed by western blot, and RNA by RT/PCR, ribonuclease protection, and multiplex RT/PCR. Myostatin cDNA and shRNA were injected, with or without TGFβ1, in the rat penile TA, and plaque size was estimated by Masson trichrome.

Results. Myostatin was detected in the human TA, and a 3-fold over-expression was found in the PD plaque. In PD cells, myostatin was mainly localized in myofibroblasts and TGFβ1 stimulated its expression, that in TA cells increased upon passage paralleling myofibroblast differentiation. Myostatin or its cDNA construct increased myofibroblast number and collagen deposition in TA cells. Myostatin was detected in the TGFβ1-induced PD-like plaque of the rat, partly in myofibroblasts, and

in the TA. Myostatin cDNA injected in the rat TA induced a PD-like plaque and intensified the TGFβ1-induced lesion, which was not reduced by myostatin shRNA.

Conclusions. Myostatin is increased in the PD plaque and its cell cultures, partly due to myofibroblast generation. Although myostatin induces a plaque in the rat TA, it does not appear to mediate the one induced by TGFβ1, thus suggesting that both proteins act concurrently in PD fibrosis, and that therapy should target their common downstream effectors.

Abstracts

Abstract G-8. Skeletal muscle-derived stem cells (MDSC) seeded on small intestinal submucosal (SIS) scaffolds stimulate vaginal repair in the rat. Ho MH, Heydarkhan S, Vernet D, Kovanecz I, Ferrini MG, Bathia NN, Gonzalez-Cadavid NF.J Urol , *179, Issue 4, Supplement 1*, #*1369, Page 468*

Objectives: The repair of vaginal tissue after vaginal vault prolapse or for correcting genital abnormalities is based on surgical techniques with graft materials that are under constant development. However, in contrast to other approaches in female pelvic reconstruction, stem cells have not yet been tested for this purpose in the vagina. We have previously reported that MDSC differentiate into smooth muscle cells **(SMC)** in the rat penile corpora cavernosa and correct erectile function. Here, we have investigated in a rat model whether: a) MDSC mounted on SIS scaffolds generate in vitro SMC and other cell types; b) express specific markers applicable to their detection upon implantation into the vagina after hysterectomy and partial vaginectomy; and c) stimulate vaginal tissue repair.

Methods. MDSC were prepared from mouse skeletal muscle by the pre-plating procedure, grown on monolayer, or polymeric scaffolds on porcine SIS, under 2.5% or 20% fetal bovine serum with or without TGFβ1 (5 ng/ml), and tested for differentiation by immunocytochemistry **(ICC)** and quantitative western blot and real time PCR. Putative MDSC markers were screened by DNA microarrays (SuperArray) followed by RT/PCR, ICC, and western blot. DMEM on SIS scaffolds were labeled with DAPI and implanted on the vagina of rats that underwent hysterectomy, using as controls intact rats and hysterectomized rats implanted or not with SIS scaffolds without MDSC. Rats were immunosuppressed with tacrolimus and sacrificed after 4 or 8 weeks. Dual immunofluorescence was used to detect MDSC differentiation and hematoxylin/eosin was used for histology.

Results. MDSC, both on monolayers and scaffolds, differentiated in vitro into SMC, as determined by α-smooth muscle actin **(ASMA)**, calponin, and smoothelin. MDSC expressed Oct-4 and myoglobin, in addition to myst 4, Nanog, Notch 3, Wnt 1, CD63, and muscle creatine kinase, as potential markers. Both DAPI fluorescence and myoglobin antibody detected MDSC implanted in the vagina, and dual DAPI/ASMA indicated their conversion to SMC at 4 weeks. Vaginal tissue repair was stimulated by MDSC on SIS, as compared to SIS alone, by differentiating into SMC and restoring normal histology at 4 and 8 weeks.

Conclusions. MDSC/SIS implants stimulate vaginal tissue repair in the rat, and autologous MDSC may constitute a promissory approach for treatment of vaginal prolapse.

Bulleted list of key research accomplishments

We have demonstrated with cultures of MDSC derived from three different sources, the Mst wt mouse, the Mst ko mouse, and the mdx mouse, that:

- Implants of the Wt MDSC can regenerate in vivo smooth muscle tissue and other cell types in injured or impaired urogenital tissue and correct the functional dysfunction created by the loss of smooth muscle cells and excessive collagen deposition, and also can convert into

myofibers in the skeletal muscle, thus showing MDSC responsiveness to in vivo paracrine modulation.

- Wt MDSC express in vitro an embryonic stem cell marker, Oct-4, and cells expressing this marker could be located in vivo in the skeletal muscle using a transgenic mouse model that detects Oct-4 expression with a reporter gene, thus opening up the possibility of using this model for further studies in muscle dystrophy.

- Wt and Mst ko MDSC proved to be unexpectedly resistant to in vitro paracrine and autocrine modulation based on effectors of the myostatin pathway, but our previous in vivo results suggest that MDSC will be responsive to factors in the tissue environment

- Myostatin role as a profibrotic agent was confirmed in vitro in a multipotent cell line, the C3H 10T1/2, and in vivo in a connective tissue containing stem cells, thus confirming the need to counteract this agent in dystrophic processes.

Reportable outcomes

A. Papers acknowledging this grant (see Appendix)

G-1. Nolazco G, Kovanecz I, Vernet D, Gelfand RA, Tsao J, Ferrini MG, Magee T, Rajfer J, Gonzalez-Cadavid NF. Effect of muscle-derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. BJU Int. 2008 Feb 21; [Epub ahead of print]

G-2. Artaza JN, Singh R, Ferrini MG, Braga M, Tsao J, Gonzalez-Cadavid NF. Myostatin promotes a fibrotic phenotypic switch in multipotent C3H 10T1/2 cells without affecting their differentiation into myofibroblasts. J Endocrinol. 2008 Feb;196(2):235-49.

G-3. Cantini LP, Ferrini MG, Vernet D, Magee TR, Qian A, Gelfand RA, Rajfer J, Gonzalez-Cadavid NF (2008) Pro-fibrotic role of myostatin in Peyronie's disease. J Sex Medic, in press

B. Abstracts and presentations related to results in the current grant

G-5. J. N. Artaza Role of Smad and Wnt signaling pathways in the fibrotic differentiationof C3H 10T (1/2) multipotent cells Induced by Myostatin", Presented at the Western American Federation for Medical Research, January 31-February 3, 2007. Sunset Center Carmel, CA, USA. Winner of the Carmel Scholar Award. Journal of Investigative Medicine, Vol 55, Issue 01, January 2007. Abstract # 61.

G-6. Ferrini MG, Cantini LP, Vernet D, Magee TR, Qian A, Gelfand RA, Rajfer J, Gonzalez-Cadavid NF Pro-fibrotic role of myostatin in Peyronie's disease. American Urological Association May 17-22, 2008, Orlando, FL

G-7. Ho MH, Heydarkhan S, Vernet D, Kovanecz I, Ferrini MG, Bathia NN, Gonzalez-Cadavid NF. Skeletal muscle-derived stem cells (MDSC) seeded on small intestinal submucosal (SIS) scaffolds stimulate vaginal repair in the rat. American Urological Association May 17-22, 2008, Orlando, FL

G-8. Nolazco G, Kovanecz I, Vernet D, Ferrini MG, Gelfand R, Tsao J, Magee T, Rajfer J, Gonzalez-Cadavid NF. Effect of muscle derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. American Urological Association May 17-22, 2008, Orlando, FL

G-9. Gonzalez-Cadavid NF. Molecular basis of Peyronie's disease. Ann Meet SMSNA, Orlando, Fl, May 2008

C. New applications for funding

The following grant applications, which are still pending or already awarded, have been submitted, by investigators in this DOD grant using in part results obtained during year 1 of this grant:

1. Nitric oxide/cGMP modulation of skeletal muscle stem cell differentiation in myocardial infarction in the mouse (PI and mentor: Gonzalez-Cadavid NF; one year student training grant: J. Wang: #0543-38F). NIH GCRC at Harbor-UCLA. Awarded December 2007
2. Regeneration of the Pelvic and Urethral Supports by Muscle Derived Stem Cells. (Mentor: Gonzalez-Cadavid NF; PI: Ho, M; 2 years). NIH SCORE/SC2. Awarded March 2008
3. Regeneration of Skeletal and Smooth Muscles by Muscle-Derived Stem Cells for the Treatment of Aging Female Pelvic Floor Disorders. (Mentor: Gonzalez-Cadavid NF; PI: Ho, M; 2 years). Jahnigen Career Development Scholars Award, American Geriatrics Society. Awarded March 2008
4. Modulation of stem cell differentiation in diabetes-related erectile dysfunction (PI: Gonzalez-Cadavid NF; 5 years). Submitted on 03/17/08 as main research grant in the O'Brien Urology Center at LABioMed Harbor-UCLA, to NIH-NIDDK in response to RFA. Pending
5. Repair of cavernosal nerve damage using stem cells and nitric oxide upregulators (PI: Ferrini, MG; 4 years)**.** Submitted on 03/17/08 as main research grant in the O'Brien Urology Center at LABioMed Harbor-UCLA, to NIH-NIDDK in response to RFA. Pending
6. Therapy of penile corporal fibrosis and erectile dysfunction in a rat model of type 2 diabetes by nitric oxide/cGMP modulation of stem cell differentiation (Co-PI: Gonzalez-Cadavid, NF; 3 years). RAICES International Cooperation Program, Argentina FONCYT. Pending
7. Modulation of skeletal muscle stem cell differentiation into cardiomyocytes (Mentor: Gonzalez-Cadavid, NF, PI: Artaza J; 4 years). Within G12RR030262 NIH RCMI Infrastructure Development grant renewal (PI: Kelly, Baker; NGC: Core Director). Submitted February 2008. Pending
8. Molecular Medicine and Stem Cells Research Core (Director: Gonzalez-Cadavid NF; PI: Kelly S/Baker R; 5 years), G12RR030262 NIH/RCMI Infrastructure Development grant Submitted February 2008. Pending

D. Appointment and Replacements

During 2007 the College of Medicine at Charles Drew University underwent a considerable reorganization, to recover from the impact of the closure of the associate Martin Luther King Hospital. Although this affected only medical services, residences and training, and basic translational research did not suffer directly, there were however some indirect consequences in terms of research program grants like the NIH RCMI renewal being postponed until the overall university situation was clarified. Productive junior research faculty had to reassess their academic and research group affiliations and interests, to try to find new funding sources that would cover most of their salaries, either as PIs or as collaborators in other PIs grants. In turn new prospects arose for junior faculty through the NIH MBRS Score Program sponsoring their path to independence. As a result, grants like the current one that would cover only a small fraction of their research effort as research associates were affected by this internal faculty mobility towards larger grants.

For these reasons, two co-investigators at the Charles Drew University site, Jorge Artaza, PhD and Rajan Singh, PhD, have switched their research work to other topics in order to pursue their own independent research careers, although Dr. Artaza will continue collaborating with the PI in another project (if funded). Therefore, they have been replaced by a combination of corrective

measures. First, by increasing the effort of the budgeted research associate, James Tsao, MD (unlicensed), BSc, from 50% to 100%. He has worked for several years with the PI and has been successfully trained in all the activities that Dr. Artaza and Singh were initially planned to carry out. Second, by assisting him through the work of Gaby Nolazco, MSc., at 20% effort. Ms Nolazco is also a long-time collaborator of the PI, who has substantial experience in the use of the MDSC. Their biosketches are enclosed in the Appendix. All the animal experiments will continue at Charles Drew University, where the IACUC has approved our protocols with animals.

At the LABioMed site there were also changes which in part reflected new recruitment by Charles Drew University, and also similar needs of junior faculty to look for their independence or more solid type of salary support. The principal investigator responsible for the LABioMed site, Monica G. Ferrini PhD has been recruited as full time faculty (Assistant Professor) at Charles Drew University, where she is pursuing her own independent research career, although she continues collaborating with the PI in other research topics. Therefore, she has resigned her LABioMed position and her site-PI role in this grant, although she may continue collaborating at CDU.

A long-term collaborator at LABioMed, Istvan Kovanecz PhD, and Assistant Professor at UCLA, has been selected to act as new PI at 20% effort at the LABioMed site, since he is very experienced in immunohistochemistry and molecular biology. His biosketch is enclosed in the Appendix. Finally, a research associate at the LABioMed site, Thomas Magee, PhD, has also been promoted within another Department at LABioMed Harbor-UCLA where he is pursuing his own independent research career, although he also continues collaborating with the PI in other research topics. Dolores Vernet PhD, who is a long time collaborator of the PI and has extensive experience with MDSC and Robert Gelfand, PhD, also a collaborator and with considerable experience in molecular biology, replaced Dr. Magee at 10% effort each one, respectively, and will continue as such.

Conclusions

This first year work, as extracted under Key Research Accomplishments and presented in brief below under Summary of Results, has provided a series of suggestions for new in vitro experiments to complement to the already planned Tasks 3 and part of 4 that are essentially in vivo, namely:

a) to detect whether the myostatin ActIIb receptor is expressed in these MDSC and their generated myotubes (it is in the C3H 10T1/2 cells) that would explain the failure of recombinant myostatin;

b) to detect whether there is a decay in myostatin expression from the Mst cDNA transfected into MDSC;

c) to check myogenesis using earlier passages that would probably impose a new generation of fresh Wt and Mst ko MDSC;

d) to run the myogenesis tests in all pP2-pP5 fractions to explain the failure of pP6 from Mst ko to generate myotubes;

e) to obtain new pP6 from Mst ko animals

f) to run all the tests on the recently obtained mdx MDSC and the pP2-pP5 fractions.

We are confident that the in vivo modulation on the cells implanted as in task 4 may be successful within the environment provided by the skeletal muscle tissue itself, and that their relative resistance to paracrine and autocrine influences observed in vitro will be overcome in the mouse muscle.

References

They are listed in the papers enclosed in the Appendix, as well below:

1. Yamanaka S, Li J, Kania G, Elliott S, Wersto RP, Van Eyk J, Wobus AM, Boheler KR. Pluripotency of embryonic stem cells. Cell Tissue Res. 2008 Jan;331(1):5-22.
2. Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol. 2008 Jan;26(1):101-6.
3. Ohmura M, Yoshida S, Ide Y, Nagamatsu G, Suda T, Ohbo K. Spatial analysis of germ stem cell development in Oct-4/EGFP transgenic mice. Arch Histol Cytol. 2004 Nov;67(4):285-96.
4.Ohbo K, Yoshida S, Ohmura M, Ohneda O, Ogawa T, Tsuchiya H, Kuwana T, Kehler J, Abe K, Schöler HR, Suda T. Identification and characterization of stem cells in prepubertal spermatogenesis in mice. Dev Biol. 2003 Jun 1;258(1):209-25.
5. Yoshimizu T, Sugiyama N, De Felice M, Yeom YI, Ohbo K, Masuko K, Obinata M, Abe K, Schöler HR, Matsui Y. Germline-specific expression of the Oct-4/green fluorescent protein (GFP) transgene in mice. Dev Growth Differ. 1999 Dec;41(6):675-84.
6. Singh R, Artaza JN, Taylor WE, (Gonzalez-Cadavid NF*, Bhasin S*; *: equal contributors) (2003) Androgens stimulate myogenenic differentiation and inhibit adipogenesis in C3H 10T1/2 pluripotent stem cells through an androgen receptor-mediated pathway. Endocrinology, 144:5081-508
7. Artaza JN, Bhasin S, Magee TR, Reisz-Porszasz S, Shen R, Groome NP, Fareez MM, Gonzalez-Cadavid NF (2005) Myostatin inhibits myogenesis and promotes adipogenesis in C3H 10T(1/2) mesenchymal multipotent cells. Endocrinology 146:3547-3557.
8. Mizuno H, Zuk PA, Zhu M, Lorenz HP, Benhaim P, Hedrick MH. Myogenic differentiation by human processed lipoaspirate cells. Plast Reconstr Surg. 2002 Jan;109(1):199-209; discussion 210-1.
9. Péault B, Rudnicki M, Torrente Y, Cossu G, Tremblay JP, Partridge T, Gussoni E, Kunkel LM, Huard J. Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. Mol Ther. 2007 May;15(5):867-77.
9'. Usas A, Huard J. Muscle-derived stem cells for tissue engineering and regenerative therapy. Biomaterials. 2007 Dec;28(36):5401-6.
10. Deasy BM, Gharaibeh BM, Pollett JB, Jones MM, Lucas MA, Kanda Y, Huard J. Long-term self-renewal of postnatal muscle-derived stem cells. Mol Biol Cell. 2005 Jul;16(7):3323-33.
11. Magee TR, Artaza JN, Ferrini MG, Zuniga FI, Cantini L, Reisz-Porszasz S, Rajfer J, Gonzalez-Cadavid NF (2006). Myostatin shRNA gene therapy increases muscle mass. J Gene Med 8:1171-81

Summary of results

The mouse MDSC, first described and so far used mainly by Huard's group in Pittsburgh **(9,10),** have shown in our hands to be a reliable adult stem cell preparation able to replicate so far indefinitely and generate in vitro multiple cell lines, particularly myofibers, with the added advantage for this proposal of not undergoing adipogenic differentiation and expressing myostatin at basal levels. Although MDSC lost their myogenic potential at late passages (about 70), this is not of concern for autologous implants where a large number of cells can be obtained at much earlier passages.

We proved that implanted MDSC were very effective in generating smooth muscle cells and other cell types in urogenital tissue regeneration and correct a functional dysfunction created by the loss of smooth muscle cells, and also myofibers in the skeletal muscle, thus showing MDSC responsiveness to in vivo paracrine modulation. We showed that MDSC express an embryonic stem cell marker, Oct-4, and that cells expressing this marker could be located in the skeletal muscle using a transgenic mouse model, thus opening up the possibility of using this model for further studies in muscle dystrophy. Moreover, the tools we intended to use in this grant, the myostatin cDNA or the antagonic myostatin shRNA adenoviral and plasmid constructs were shown to induce fibrogenic

differentiation or blocking it in vitro in the C3H 10T1/2 multipotent cell and in vivo in a connective tissue containing stem cells.

However, to our surprise MDSC proved to be quite resistant to in vitro paracrine modulation, since myogenic differentiation was not reduced by recombinant myostatin (an inhibitor of this process in C3H 10T1/2 cells). Even more unexplainable so far, the MDSC from the myostatin ko mouse were unable to undergo myogenesis. Similarly, autocrine modulation by transfection with myostatin cDNA or the antagonic myostatin shRNA adenoviral and plasmid constructs did not significantly affect myogenesis in the wild type MDSC or restore it in the Mst Ko MDSC. The two myostatin-related agents that were able to moderately modulate in vitro MDSC myogenesis were follistatin and TGFβ1, since not even testosterone (that increase myogenesis in C3H 10T1/2 cells) affected their myogenic commitment and only their fusion to induce hypertrophy.

Appendices

They include:

1) The downloaded publications for papers G1-G3
2) The biographical sketches of Gonzalez-Cadavid, Ferrini, Kovanecz, Vernet, Gelfand, and Nolazco-Gomez

BJU INTERNATIONAL

EFFECT OF MUSCLE DERIVED STEM CELLS ON THE RESTORATION OF CORPORA CAVERNOSA SMOOTH MUSCLE AND ERECTILE FUNCTION IN THE AGED RAT

Journal:	*BJU International*
Manuscript ID:	BJU-2007-1076
Manuscript Type:	Original Article
Date Submitted by the Author:	09-Oct-2007

Complete List of Authors:	Nolazco, Gaby; Los Angeles Biomedical Research Institute, Urology Kovanecz, Istvan; Los Angeles Biomedical Research Institute, Urology Vernet, Dolores; Los Angeles Biomedical Research Institute, Urology Gelfand, Robert; Los Angeles Biomedical Research Institute, Urology Tsao, James; Charles R Drew University, Endocrinology Ferrini, Monica; Los Angeles Biomedical Research Institute, Urology; David Geffen School of Medicine at UCLA, Urology Magee, Thomas; Los Angeles Biomedical Research Institute, Urology; Harbor-UCLA Medical Center, Urology; David Geffen School of Medicine at UCLA, Urology Rajfer, Jacob; Los Angeles Biomedical Research Institute, Urology; Harbor-UCLA Medical Center, Urology; David Geffen School of Medicine at UCLA, Urology Gonzalez-Cadavid, Nestor; Los Angeles Biomedical Research Institute, Urology; Charles R Drew University, Endocrinology; Harbor-UCLA Medical Center, Urology; David Geffen School of Medicine at UCLA, Urology
keywords:	erectile dysfunction, corporal fibrosis, CVOD

Abstract:	Objective To determine whether skeletal muscle derived stem cells (MDSC) convert into smooth muscle cells (SMC) both in vitro and in vivo, and in so doing ameliorate the erectile dysfunction of aged rats, and whether endogenous stem cells are present in the rat corpora cavernosa. Materials and Methods MDSC were obtained from mouse muscle, and shown by immunocytochemistry for α-smooth muscle actin (αSMA) to originate in vitro myofibroblasts and SMC, discriminating SMC by calponin 1 expression. In vivo: these MDSC, labeled with 4',6-Diamidino-2-phenylindole (DAPI) were implanted

into the corpora cavernosa of young adult (5 month old) and aged (20 month old) rats for 2 and 4 weeks. Histological changes were assessed by immunohistochemistry and quantitative western blot. Functional changes were determined by electrical field stimulation (EFS) of the cavernosal nerve.
Results
The exogenous cells replicated and converted into SMC, as shown in corporal tissue sections by confocal immunofluorescence microscopy for proliferating cell nuclear antigen (PCNA), αSMA, and smoothelin, and also by western blot for αSMA and PCNA. MDSC differentiation was confirmed by the activation of the αSMA promoter-linked β-galactosidase in transfected cells, both in vitro and after implantation in the corpora. Putative endogenous stem cells were shown in corporal tissue sections and western blots by detecting CD34 and a possible Sca1 variant. EFS showed that implanted MDSC raised in aged rats the maximal intracavernosal pressure/mean arterial pressure levels above (2 weeks) or up to (4 weeks) those of young adult rats.
Conclusions
MDSC implanted into the corpora cavernosa of aged rats converted into SMC and corrected erectile dysfunction, and endogenous cells expressing stem cell markers were also found in the untreated tissue. This suggests that exogenous stem cell implantation and/or endogenous stem cell modulation may be viable therapeutic approaches for aging-related erectile dysfunction.

EFFECT OF MUSCLE DERIVED STEM CELLS ON THE RESTORATION OF CORPORA CAVERNOSA SMOOTH MUSCLE AND ERECTILE FUNCTION IN THE AGED RAT

Nolazco G*, Kovanecz I*, Vernet D*, Gelfand RA*, Tsao J¶, Ferrini M*‡, Magee T*†‡, Rajfer J*†‡, Gonzalez-Cadavid NF*†‡¶

*Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Urology Research Laboratory, †Division of Urology, Harbor-UCLA Medical Center, Torrance, CA, ‡Department of Urology, David Geffen School of Medicine at UCLA, Los Angeles, CA, and ¶Department of Internal Medicine, Division of Endocrinology, Charles R Drew University

Corresponding author: Nestor F. Gonzalez-Cadavid, Ph.D., Harbor-UCLA Medical Center, Urology, Bldg. F-6, 1000 West Carson Street, Torrance, CA 90509

Telephone: (310)-222-3824; Fax: (310)-222-1914; **e-mail**: ncadavid@ucla.edu

Running Title: Stem cells, smooth muscle, and erectile function

Key words: erectile dysfunction, corporal fibrosis, CVOD;

Abbreviations: αSMA, α-smooth muscle actin; **αSMAPr-β-gal**, plasmid construct expressing the E. coli β-galactosidase gene under the rat αSMA promoter; **CVOD**, corporal veno-occlusive dysfunction; **DAPI**, 4',6-Diamidino-2-phenylindole; **DMEM**, Dulbecco's Modified Eagle's Medium; **EFS**, electrical field stimulation of the cavernosal nerve; **FBS**, fetal bovine serum; **MAP**, mean arterial pressure; **MDSC**, skeletal muscle derived stem cells; **MHC-II**, myosin heavy chain 2**;** **MIP**, maximal intracavernosal pressure; **PCNA**, proliferating cell nuclear antigen **pP6**, preplate fraction 6 (MDSC); **SMC**, smooth muscle cells; **TGFβ1**, transforming growth factor β1**.**

Summary

Objective

To determine whether skeletal muscle derived stem cells (MDSC) convert into smooth muscle cells (SMC) both in vitro and in vivo, and in so doing ameliorate the erectile dysfunction of aged rats, and whether endogenous stem cells are present in the rat corpora cavernosa.

Materials and Methods

MDSC were obtained from mouse muscle, and shown by immunocytochemistry for α-smooth muscle actin (αSMA) to originate in vitro myofibroblasts and SMC, discriminating SMC by calponin 1 expression. In vivo: these MDSC, labeled with 4',6-Diamidino-2-phenylindole (DAPI) were implanted into the corpora cavernosa of young adult (5 month old) and aged (20 month old) rats for 2 and 4 weeks. Histological changes were assessed by immunohistochemistry and quantitative western blot. Functional changes were determined by electrical field stimulation (EFS) of the cavernosal nerve.

Results

The exogenous cells replicated and converted into SMC, as shown in corporal tissue sections by confocal immunofluorescence microscopy for proliferating cell nuclear antigen (PCNA), αSMA, and smoothelin, and also by western blot for αSMA and PCNA. MDSC differentiation was confirmed by the activation of the αSMA promoter-linked β-galactosidase in transfected cells, both in vitro and after implantation in the corpora. Putative endogenous stem cells were shown in corporal tissue sections and western blots by detecting CD34 and a possible Sca1 variant. EFS showed that implanted MDSC raised in aged rats the maximal intracavernosal pressure/mean arterial pressure levels above (2 weeks) or up to (4 weeks) those of young adult rats.

Conclusions

MDSC implanted into the corpora cavernosa of aged rats converted into SMC and corrected erectile dysfunction, and endogenous cells expressing stem cell markers were also found in the

untreated tissue. This suggests that exogenous stem cell implantation and/or endogenous stem cell modulation may be viable therapeutic approaches for aging-related erectile dysfunction.

INTRODUCTION

Aging related erectile dysfunction is primarily due to corporal veno-occlusive dysfunction (CVOD) [1-2], as a result of a loss of the corporal smooth muscle cells (SMC) together with excessive collagen deposition within the corpora, as shown both in man [3-5] and rat models [6-14]. It has been hypothesized that this histological alteration is due to oxidative stress triggered by the release of profibrotic factors such as reactive oxygen species**,** transforming growth factor β1 (TGFβ1), plasminogen activator inhibitor-1, and others, that not only leads to collagen accumulation but also to an increase in apoptosis and a reduction in corporal SMC proliferation [6-14]. As such, it appears as if the ideal way to treat this aging related erectile dysfunction would be to reverse or prevent these changes, because such an approach has the potential to become a curative rather than a palliative intervention for this form of erectile dysfunction.

Recently, it has been demonstrated experimentally in the aged rat that the long-term and sustained administration of PDE5 inhibitors will not only increase the SMC to collagen ratio within the corpora cavernosa but will improve also the underlying CVOD in these animals [6]. Such a pharmacological approach has been used successfully in both the rat and man with another form of erectile dysfunction, the post prostatectomy animal model and the post prostatectomy patient [4,9,15,16]. In both species, the long-term administration of PDE5 inhibitors resulted in preservation of the normal SMC to collagen ratio within the corpora.

While it is possible that pharmacological therapy, or even gene therapy [17], may one day prove to be efficacious in reversing aging related and other forms of erectile dysfunction in the human, it has recently been reported that the therapeutic applications of stem cells [18,19] may extend to the replenishment of the corporal SMC population that is impacted by the aging process. To date, only a few studies of stem cell implantation in experimental models of erectile dysfunction, to repair either nerves or smooth muscle in the corpora, have been conducted [20-

23], although stem cells are also being investigated for the repair of other urogenital organs, such as the bladder, urethra, and kidney [24-27]. The first study in the penis [20] was based on the injection of rat embryonic stem cells modified ex vivo to express brain derived nerve growth factor into the corpora cavernosa in a rat model of cavernosal nerve damage. Although there was an improvement in erectile function and neurofilament staining even at 3 months after injection of the stem cells, no surviving stem cells were found nor were any SMC markers investigated, since the primary objective of this study was to achieve nerve regeneration. A more recent report did claim that implantation of human bone marrow mesenchymal stem cells into the normal rat corpora resulted at two weeks in the differentiation of these stem cells into both endothelial and smooth muscle cells, but retrovirus-immortalized cells and not truly self-replicating cells were used [21]. A more convincing study was performed with native rat bone marrow mesenchymal stem cells engineered to express endothelial nitric oxide synthase (eNOS), where these stem cells were injected into the corpora cavernosa of aged rats [22]. Not only was erectile dysfunction corrected, but SMC markers were expressed at 3 weeks after implantation.

Stem cells isolated from the adult skeletal muscle, or “muscle-derived stem cells” (MDSC), have been investigated extensively because of their active prolonged proliferation, low immunogenicity, and ability to convert into several cell lineages after implantation into different organs [28]. MDSC are not to be confused with regular myoblast or “satellite cell” preparations. These types of cells are not pluripotent but have been used in clinical trials for treating heart disease since satellite cells may generate active syncitia with cardiomyocytes, or even new cardiomyocytes [29]. MDSC may be prepared from skeletal muscle biopsies, more accessible for autologous transplants than bone marrow, and as such do not pose the immunogenic risks of embryonic stem cells [28]. MDSC have been claimed to generate SMC in vitro [30], albeit the SMC characterization was not performed with true SMC markers, such as calponin or smoothelin and particularly in vivo after implantation in the urethra in a rat model of stress

urinary incontinence [26,31]. Intrapenile injection of MDSC also improved erectile function in a bilateral cavernosal nerve resection rat model of erectile dysfunction [23], but since the objective was to improve nerve regeneration after bilateral cavernosal nerve resection, no studies on MDSC differentiation into corporal SMC were performed.

From the above it follows that MDSC may be preferable to other stem cells for potential clinical applications aimed at restoring functional SMC in the corpora cavernosa, particularly in aging-related erectile dysfunction where, as stated, CVOD is the main manifestation**.** However, the MDSC to SMC differentiation in this context requires further biological characterization and the demonstration that it is functionally effective in a related animal model. Moreover, since we previously characterized stem cells in the human penile tunica albuginea that were able to generate SMC [32], it is important to determine whether stem cells are also detectable among the SMC in the corpora cavernosa itself, which could potentially be activated by the paracrine effects of the exogenous implant, or by pharmacological interventions. In the present work we have implanted MDSC into the corpora in the aged rat model, and studied both their effects on the erectile response to electrical field stimulation of the cavernosal nerve (EFS), and on their replicative and differentiation ability utilizing immunoblotting, confocal microscopy, and activation of a gene promoter for a SMC marker. We also investigated whether endogenous stem cells are present in the rat penis, specifically in the corpora cavernosa.

MATERIALS AND METHODS

Skeletal muscles were obtained from the hind limb of C57BL/6 mice and MDSC were isolated applying the preplating procedure [33]. The mouse skeletal mouse was preferred because these MDSC are the only ones prepared by this method that have been extensively characterized as stem cells [28], whereas isolating them from rat skeletal muscle would require us to validate this procedure on the rat cells. Briefly, tissues were dissociated using sequentially collagenase XI, dispase and trypsin, and after filtration through 60 nylon mesh, and pelleting, the released cells

were suspended in GM-20 (Dulbecco's Modified Eagle's Medium (DMEM)/20% fetal bovine serum (FBS). Cells were then plated onto collagen I-coated flasks for 2 hrs (preplate 1 or pP1), followed by a series of sequential daily decantation of floating cells and platings for 2 to 6 days, until preplate 6 (pP6). The latter is the cell population containing MDSC. Fibroblasts are concentrated in the preplate 2 (pP2) fraction while satellite cells are essentially in the pP3 and pP4 fractions. Cells were counted in each supernatant. In general, cells were maintained in DMEM/20% fetal bovine serum (FBS) on regular culture flasks (no coating) and used in the 15-20th passage, since MDSC from mouse muscle were properly characterized with stem cell markers have been maintained in our laboratory for at least 40 generations with the same, or even increasing, growth rate. The absence of SMC in these enriched stem cells was verified at the initial passages by immunocytochemistry and western blot for α-smooth muscle cell actin (αSMA).

For in vitro experiments involving the activation of the αSMA gene promoter to detect SMC generation, MDSC were grown onto 6 well plates and transfected at 80% confluence with a construct expressing the β-galactosidase gene under this promoter (αSMA Pr-β-gal). The construct was prepared by substituting the rat αSMA 764 bp promoter region [41] (Genbank S76011.1), modified to include Eco RI and Asc I restriction sites at its 5' end and a Xho I site at its 3' end, for the CMV promoter of the pCMVβ plasmid (Clontech, Mountain View, CA, USA) upstream from the full length E. coli β-galactosidase gene. On the following day, cells were transferred to DMEM with 2.5% FBS and TGFβ1 at 5 ng/ml to induce differentiation, and maintained for 6 days. Cells were then fixed with 1% glutaraldehyde, stained with X-gal [35], and counterstained with fast red. For in vivo implantation, MDSC were transfected similarly and cultured for 7 days in DMEM/20% FBS.

Male Fisher 344 rats (Harlan Sprague-Dawley Inc., San Diego, CA, USA), of 20 months of age ("aged") were used for EFS determinations and some histological/biochemical detections (8/group/period), while we used 5 month old animals ("young adult") only for some preliminary

non-EFS assays (4/period). The animals were treated according to National Institutes of Health regulations with an Institutional Animal Care and Use Committee-approved protocol. MDSC ($0.5\text{-}1.0x10^6$ cells/50 µl Hanks) were labeled with the nuclear fluorescent stain 4',6-Diamidino-2-phenylindole (DAPI) and implanted aseptically into two different sites in the mid part of the shaft in anesthetized animals. Tacrolimus was given daily (1 mg/kg, sc) to avoid immuno rejection of the mouse stem cells. At 1, 2, and 4 weeks after implantation, rats were either killed (1, 2, 4 weeks, young adult rats only), or subjected to EFS and then killed (2 and 4 weeks, aged rats only). An additional group of four young adult rats was similarly implanted with MDSC transfected with the αSMA-β galactosidase construct and killed at 5 and 10 days. For tissue excision, animals were perfused with saline under anesthesia, killed, then the penises were excised and denuded, and small portions of the penile shaft tissue were cryoprotected in 25% sucrose, immersed in OCT, and subjected to cryosectioning (5 µm for regular microscope, or 20-30 µm for confocal microscope) without fixation. The remainder of the penile shaft tissue was frozen in liquid nitrogen and stored at –80 °C.

Cells on collagen-coated 8 well removable chambers, and frozen tissue sections, were reacted [8,9,10,15,16,32] with some of the following primary antibodies against: 1) human myosin heavy chain type II (MHC-II) (monoclonal, 1:200 Vector Laboratories, Burlingame, CA, USA) a marker for skeletal myotubes), 2) human αSMA (mouse monoclonal in Sigma kit, 1:2, Sigma Chemical, St Louis, Mo, USA) a marker for both SMC and myofibroblasts, 3) human calponin-1 (basic) (mouse monoclonal, 1:25, Novocastra, Burlingame, CA, USA), and 4) chicken smoothelin (mouse monoclonal, 1:100, Abcam, Cambridge, Ms, USA), two exclusive markers for SMC, 5) proliferating cellular nuclear antigen (PCNA) (mouse monoclonal, 1:100, Chemicon, Temecula, CA, USA) a marker for replicating cells, 6) human CD34 (rabbit polyclonal, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), a stem cells marker, and 7) mouse Sca1 (rat monoclonal, 1:200, BD Pharmigen, Franklin Lake, NJ, USA) another stem cell marker.

For cells not previously labeled with DAPI, cultures or tissue sections were subjected to immunohistochemical detection by quenching in 0.3% H_2O_2-PBS, blocking with goat (or corresponding serum), and incubated overnight at 4 °C with the primary antibody. This was followed by biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA), respectively, for 30 min, the ABC complex containing avidin linked horse radish peroxidase (1:100; Vector), 3,3' diaminobenzidine (DAB), and counterstaining with hematoxylin, or no counterstaining.

For cells labeled with DAPI, fluorescent detection techniques were used. The secondary anti-mouse IgG antibody was biotinylated (goat, 1:200, Vector Laboratories) and this complex was detected with streptavidin-Texas Red. For the CD34 detection we used a biotinylated anti-rabbit IgG (goat, 1:200, Vector Laboratories). After washing with PBS, the sections were mounted with Prolong antifade (Molecular Probes, Carlsbad, CA, USA). Negative controls in all cases omitted the first antibodies or were replaced by IgG isotype.

Sections were viewed under an Olympus BH2 fluorescent microscope or in a confocal using blue and/or red filters and overlay. Confocal fluorescence images were taken on a Leica TCS-SP Confocal Microscope (Heidelberg, Germany), equipped with an argon laser (488 nm blue excitation: JDS Uniphase), a diode laser (DPSS; 561 nm yellow-green excitation): Melles Griot), a helium-neon laser (633 nm (red). Spectral emission filters were set at 500-550 nm for green fluorescence and 580-700 nm for red fluorescence. Cytochemistry for alkaline phosphatase to detect osteoblasts in vitro was performed as described [32]. For quantitative image analysis, staining intensity was determined by computerized densitometry using the ImagePro Plus 5.1 program (Media Cybernetics, Silver Spring, MD, USA), coupled to the Olympus BH2 microscope with a Spot RT color digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). The number of positive cells was expressed as a percentage of the total cells. In all cases, five non-overlapping fields were screened per well.

For western blots [8,9,10,15,32], cell homogenates were obtained in boiling lysis buffer

(1% SDS, 1mM sodium orthovanadate, 10 mM Tris pH 7.4 and protease inhibitors: 3 μM leupeptin, 1 μM pepstatin A, 1mM phenylmethylsulphonyl fluoride), and centrifuging at 16,000 *g* for 5 min. 30 μg of protein were run on 7.5% or 10% polyacrylamide gels, and submitted to western blot and immunodetection with the antibodies against PCNA, CD34, and Sca1 described above, or with an antibody against human αSMA (monoclonal, 1:1000, Calbiochem, La Jolla, CA, USA). Membranes were incubated with a secondary polyclonal horse anti-mouse IgG linked to horseradish peroxidase (1:2000; BD Transduction Laboratories, Franklin Lakes, NJ, USA, or 1:5000, Amersham GE, Pittsburgh, PA, USA) and bands were visualized with luminol (Pierce, Rockford, IL, USA). In the case of Sca1, the secondary peroxidase-linked antibody was anti-rat IgG (rabbit, 1:2000, Sigma Chemical). For the negative controls the primary antibody was omitted.

EFS in the rat was performed as described [36,37]. Briefly, under anesthesia, the cavernosal nerve was exposed, and hooked by a bipolar platinum electrode. Systemic arterial and intracavernosal pressure measurements were obtained by simultaneous intrafemoral artery and cavernosal catheterization, respectively. EFS was applied at 10 V and a frequency of 15 Hz for pulses of 60 sec, separated by 2-min intervals, with a Grass Stimulator (Grass Instruments Co., Quincy, MA). A data acquisition system (Biopac Systems, Santa Barbara, CA) simultaneously recorded arterial blood and intracavernosal pressure, and values were expressed in mmHg. The ratio between the maximal intracavernosal pressure (MIP) and the mean arterial pressure (MAP) at the peak of erectile response were calculated, to normalize for variations in blood pressure.

Values are expressed as the mean (SEM). The normality distribution of the data was established using the Wilk–Shapiro test. Multiple comparisons were analyzed by a single factor ANOVA, followed by *post hoc* comparisons with the Newman–Keuls test. Differences among groups were considered statistically significant at $p<0.05$.

RESULTS

The mouse MDSC obtained by the pre-plate procedure were about 1% of the original mononucleated cell population extracted from the normal mouse skeletal muscle and about 60% bound to Sca1-coated magnetic beads, indicating that this cell fraction expressed the stem cell marker Sca1 (not shown). When the total non-Sca1 selected MDSC were incubated for 3 weeks in myogenic differentiation medium, they underwent conversion into multinucleated skeletal myotubes that express MHC-II and are equivalent to the skeletal myofibers (Fig. 1-A). The myogenically committed precursors, the satellite cells [28], were excluded in the initial platings by their faster adhesion to the culture flasks. MDSC were also able to convert into myofibroblasts and osteoblasts when they were incubated respectively in fibrogenic medium, containing TGFβ1 for 2 weeks, as detected by αSMA (which also is expressed in SMC) combined with the typical morphology for the actin filaments (B), or in osteogenic medium for 4 weeks, as detected by alkaline phosphatase (C). MDSC conversion into SMC was assessed for 4 weeks in DMEM-2.5% fetal bovine serum and 20 nM rapamycin to enhance the contractile phenotype [51] by another SMC marker that is not expressed in myofibroblasts, namely calponin. In this case, MDSC were labeled with DAPI and calponin identified by immunodetection with Texas red fluorescence, observing the positive cells in magenta color in the overlay of the blue and red filters (D). Although only a few cells per field intensively expressed calponin, many others had a faint or moderate expression. Quantitative image analysis (E) showed substantial skeletal myotube formation by MDSC (about 8 per field), as well as 20% conversion into myofibroblasts and/or SMC, and 80% into osteoblasts. SMC were not quantitated because of the variable levels of calponin expression in the fields.

The MDSC were then labeled with DAPI, and injected into the corpora cavernosa of young adult rats, that were immunosuppressed with tacrolimus to prevent a potential inter-species immunorejection, and killed at 1, 2, and 4 weeks after implantation. Representative pictures of cryosections around the site of injection at 2 weeks showed the presence of

abundant DAPI-labeled MDSC around the corpora cavernosa cisternae that were inserted in between endogenous cells whose nuclei were propidium iodide positive but DAPI negative (Fig. 2, A vs. B). The merge (overlay) of both images shows in magenta the implanted MDSC nuclei (C). In other fields (not shown), a much lower fraction of the MDSC nuclei were present (DAPI+) among the propidium iodide labeled total nuclei. Not all the implanted MDSC were in active replication, as indicated by the comparison of DAPI stained nuclei (D) with cells immunostained for PCNA, a marker of proliferation, that were concentrated in a certain region (E, F). Essentially the same was observed at 1 week, and replicative cells were also seen at 4 weeks (not shown).

The conversion of implanted MDSC into SMC that line up the corpora cavernosa cisternae was suggested by αSMA staining, using a fluorescent confocal microscope that produces optical sections of 0.5 μm or less thickness. This allowed colocalization of the implanted nuclei after a longer period (4 weeks) (Fig 3, top A,C) with the αSMA positive cells (B), thus eliminating potential artifacts from overlapping planes. Confirmation was obtained by immunofluorescence staining with smoothelin, a specific SMC marker, which in addition to calponin is not expressed in myofibroblasts that showed many magenta cells concentrated around the cisternae in the overlay (Fig. 3, top D, F vs. E). MDSC implantation increased the αSMA content of the penile shaft tissue, assayed by western blot, as compared with penises that were not implanted with cells (bottom), although a larger n is required to confirm this visual assessment. No stimulation of cell proliferation, as indicated by PCNA, was however observed in the injected tissues.

MDSC conversion into SMC involves transcriptional activation of genes related to the contractile phenotype, such as αSMA, and this process is a direct indicator of the generation of SMC and potentially of myofibroblasts. We constructed a plasmid expressing a reporter gene (β-galactosidase) under the control of the αSMA promoter, and transfected separately cultures of rat SMC (Fig. 4, A) and of mouse MDSC (B), which were then incubated for 7 days with TGFβ1 as differentiation factor [38,39]. β-galactosidase was detected histochemically in blue

staining, and all nuclei were identified by fast red counterstain. Only a small fraction of morphologically distinctive SMC exhibited activation of the αSMA promoter, probably as a reflection of most cells being already in the contractile phenotype, with little de novo αSMA activation (A). In the case of MDSC, since only a fraction of cells were expected to differentiate, and the period of incubation was short, αSMA activation was confined therefore to even fewer cells (B). In vivo, the injection of DAPI-labeled MDSC into the corpora cavernosa for 5 and 10 days, identified by blue fluorescent nuclei (C), was accompanied by the appearance of some histochemically detected blue cells in the smooth muscle region of the corpora (D). In this case, no overlay was possible since the glutaraldehyde fixation or the beta gal histochemical staining quenches DAPI. Therefore, images are from the same well/section but not from identical sites.

The ability of the corpora cavernosal tissue to stimulate the differentiation of exogenous stem cells, such as the MDSC, suggests that this could also be the case with endogenous stem cells that may be present in the adult penis, and that may be activated for a differentiation process by the paracrine effects of exogenous MDSC. We have previously identified stem cells in fibroblast cultures from the human tunica albuginea, which generated SMC in vitro [32]. Sca1 and CD34, two markers expressed in MDSC, with the latter also previously detected in tunical multipotent cells [33], were also detected in corpora cavernosa tissue sections obtained from young rat penises that were not implanted with MDSC. This was demonstrated first for CD34 by immunohistochemistry, where many positive cells were distributed all along the tunica albuginea (solid arrows) interspersed with a few CD34 negative cells (broken arrows) (Fig. 5, top left). They were also identified in scattered clusters in the corpora cavernosa (top right). Immunofluorescence using a secondary antibody linked to Texas red confirmed this extensive distribution in the tunica (middle left) contrasted with only a few in the corpora (middle right). Western blot analysis of penile tissue homogenates from young and old rats showed the presence of the expected 97 kDa band (bottom panel).

In contrast, Sca1 was negative in the tunica albuginea and showed some positive cells along the corporal cisternae and the arterial media, both by immunohistochemistry (Fig. 6 top left) and immunofluorescence (top right). However, the expected 18 kDa Sca1 band was only visible in extracts from low passage Sca1 positive MDSC that had been selected with immunobeads carrying the Sca1 antibody. The high passage non-selected MDSC used in this work had lost completely this antigen upon culture, as shown by the absence of any band, and remarkably, the penile shaft homogenates, both from young and old rats, displayed an approximately 36-38 kDa band assumed to be a dimer of the typical Sca1 protein (bottom panel).

The therapeutic efficacy of the MDSC implanted into the corpora was assessed by a functional determination. Aged male rats (20 month old), that are an accepted model of erectile dysfunction, and specifically CVOD [6,8,36,37], were injected with 0.5-1x10^6 MDSC, and received tacrolimus, as above, for 2 and 4 weeks. Control rats received saline. The measurement of erectile function was conducted by electrical field stimulation of the cavernosal nerve (EFS) [36,37]. When compared to the control untreated old rats (Fig. 6 A) the MIP/MAP ratio in the aged rats, 2 weeks following implantation of the MDSC (B), was significantly increased to 1.13 (0.2), a value above the one usually seen in adult rats 0.8 (0.05) [36,37,40-42]. In two of these MDSC-implanted rats, the MIP was up to surprisingly 125 mm Hg, a value rarely seen in this species. When another series of rats was tested after 4 weeks of MDSC transplantation (C), although the mean MIP/MAP decreased to 0.72 (0.05), this ratio was still significantly higher than in the untreated old animals and close to that seen in 5 month old adult rats. In all these treated old rats, both at 2 and 4 weeks, DAPI positive MDSC were detected in the penile corpora cavernosa, and they were in active replication and differentiating into SMC (not shown).

DISCUSSION

Our study demonstrates that MDSC may convert into SMC when implanted into the rat corpora cavernosa and can correct aging-related erectile dysfunction for at least several weeks following implantation. The study confirms the potential efficacy of stem cells to replace the cavernosal SMC that is lost or functionally damaged in the penis during the aging process and by so, it appears to restore the normal compliance of the tissue. These findings agree with the ones recently reported using bone marrow stem cells and achieved comparable results [22].

The relatively low in vitro conversion of the MDSC into SMC was compensated by the reasonable differentiation efficacy observed in vivo, possibly because of the influence by paracrine factors secreted by the corporal tissue. In addition, since skeletal muscle biopsies are easier to obtain than other sources of stem cells, our results suggest that they may become an alternative supply of implantable cells more acceptable to patients with erectile dysfunction, if stem cells are eventually used to treat impotence, specifically the myopathy that occurs with aging. Adult autologous stem cells from different sources are already being tested in clinical trials for other conditions (see list under "stem cells" in www.clinicaltrials.gov), and even if they are less efficient than embryonic stem cells, they pose lower immunogenic and carcinogenic risks.

Our results also suggest that endogenous stem cells may be present in the penile corpora cavernosa, thus extending our previous demonstration of these multipotent cells in the human tunica albuginea [32]. We have now seen that many, if not most, cells in the tunica are CD34 positive, which suggests that this marker is also expressed in fibroblasts, the most predominant cell type in this tissue, and possibly in the stem cells previously identified by differentiation assays similar to the ones applied now to MDSC. Confirming our previous results, no Sca1 positive cells were identified in the tunica. However, in the corpora both markers were detected, albeit in just a few cells, and in the case of Sca1 the size of the protein suggests an isoform or dimer. It remains to be established whether both markers in the corpora are

expressed in the same cells, like the low passage MDSC, and whether cells expressing Sca1 and/or CD34 are indeed multipotent like their tunical counterpart. If they are, this may open up a complementary approach to MDSC implantation, by the potential modulation of endogenous stem cells to achieve similar effects. Interestingly, both nitric oxide and cGMP, physiological mediators of penile erection whose levels can be increased pharmacologically, have been shown to trigger stem cell differentiation [43-45], and therefore they may constitute new approaches to awaken dormant stem cells.

Since αSMA is also a marker of myofibroblasts, the use of confocal microscopy, with its ability to discriminate between overlapping planes and thus excluding artifacts, in combination with the detection of calponin and smoothelin as SMC markers and the in vitro and in vivo activation of the αSMA promoter in transfected cells, provides a more precise confirmation that the MDSC converts into SMC than by simply using αSMA detection and regular microscope alone. This is important, because by αSMA being also a marker of myofibroblasts, involved in penile fibrosis, the risks of inducing this cell type must be considered [46-47]. Further work is needed to conclusively exclude in vivo this possibility. Even with this caveat, our validation of MDSC conversion into SMC supports the use of MDSC for aging-related erectile dysfunction, in a fashion similar to their postulated use for the regeneration of SMC in the bladder and urethra [25,26,31], albeit if the latter was essentially based on αSMA detection. Our results seem also to be the first demonstration of MDSC in vitro conversion into SMC based on a true SMC marker, since a previous study used only ASMA [30].

This work, taken together with the previous study with bone marrow stem cells tested in the animal model of aging-related erectile dysfunction [22], establishes the proof of concept for the efficacy of stem cell therapy in regenerating SMC that undergo apoptosis in the corpora cavernosa during aging and other conditions, and thus for ameliorating vasculogenic erectile dysfunction. It may even be assumed that other cell lineages, e.g., nerves, endothelium, may be also generated by MDSC, and thus would make up for the relative loss of nitrergic nerves in the

corpora cavernosa [48] and some degree of endothelium damage in this tissue [49] that occur in the corpora during aging. The multipotency of the MDSC may therefore be a plus in certain erectile dysfunction conditions where not only SMC are lost, but there is nerve damage, such as in rat models of erectile dysfunction in diabetes [10] and post-radical prostatectomy [7,9,13,14,15,16]. In the latter case, both embryonic stem cells and MDSC themselves have shown initial promise [20,23].

Despite this encouraging proof of concept, as in the field of gene therapy [17] further animal studies are needed before attempting to transplant stem cells in the clinic, and MDSC in particular, for the therapy of erectile dysfunction. First, when using the rat as host, a truly allogenic source of MDSC has to be tested, e.g., rat MDSC, to eliminate the need for pharmacological immunosuppression or an immunodeficient strain. Second, in our study, as well as the other parallel work with bone marrow cells [22], the effects were comparatively short-lived, since in our case, the considerable stimulation of erectile function achieved at 2 weeks was reduced at 4 weeks, albeit still remaining at the normal levels of an adult rat. This reduction in MIP/MAP ratio over time may be due to the fact that one animal in this group had evidence of a preexistent testis tumor and was also adversely affected by tacrolimus. Therefore, new research has to be conducted to improve the survival of these cells and assure that the salutary functional effects can be prolonged for at least 3 to 6 months to exclude transient amelioration. Third, the detection of donor cells in the corporal tissue at these long-term treatments would require the use of stable markers, such as cells from transgenic rats expressing gfp, or selected new exogenous cell markers in lieu of fluorescent tags that are diluted along cell proliferation.

Despite these hurdles, stem cell therapy of erectile dysfunction, specifically with MDSC, is a promising approach, particularly combined with ex vivo gene transfer, as achieved previously with eNOS [22]. The list of potential genes is as extensive as in the case of non cell-mediated gene therapy, but in this case, cell modification may involve strategies to prevent undesirable myofibroblast generation and thus fibrosis. Alternatively, the engineering may aim

to intensify the differentiation capacity of stem cells into the main desired cell line, such as SMC, without reducing their multipotency to form simultaneously ancillary cell types, such as endothelial or neural cells according to needs. Another strategy would involve combination pharmacological, or gene therapy, treatment to enhance stem cell survival, e.g., by stimulating trophic factors and access to nutrients through improving a potentially defective angiogenesis in aged animals [50]. However, perhaps the greatest challenge would be to engineer by gene transfer exogenous stem cells to paracrinely awake the dormant stem cells in the penis and generate damaged or lost differentiated cells, or to directly stimulate pharmacologically a similar commitment for the endogenous stem cells.

ACKNOWLEDGEMENTS

This work was supported by grants NIH R01DK-53069, Department of Defense PC061300 and in certain aspects, NIHG12RR-03026. Dr. Ferrini and Dr. Magee are also supported by Grant N1 P20 MD000545 from the National Center on Minority Health and Health Disparities, NIH.

REFERENCES

1. Rajfer J, Rosciszewski A, Mehringer M. Prevalence of corporeal venous leakage in impotent men. J Urol 1988; **140**:69-71

2. Mulhall JP, Slovick R, Hotaling J, Aviv N, Valenzuela R, Waters WB, Flanigan RC. Erectile dysfunction after radical prostatectomy: hemodynamic profiles and their correlation with the recovery of erectile function. J. Urol 2002; **167**: 1371-5

3. Iacono F, Giannella R, Somma P, Manno G, Fusco F, Mirone V. Histological alterations in cavernous tissue after radical prostatectomy. J Urol 2005; **173**:1673-6

4. Schwartz EJ, Wong P, Graydon RJ: Sildenafil preserves intracorporeal smooth muscle after radical retropubic prostatectomy. J Urol 2004; **171**:771-4

5. Yaman O, Yilmaz E, Bozlu M, Anafarta K. Alterations of intracorporeal structures in patients with erectile dysfunction. Urol Int 2003; **71**:87-90

6. Ferrini MG, Kovanecz I, Sanchez S, Vernet D, Davila HH, Rajfer J, Gonzalez-Cadavid NF. Long-term continuous treatment with sildenafil ameliorates aging-related erectile dysfunction and the underlying corporal fibrosis in the rat. Biol Reprod 2007; **76:**915-23

7. Podlasek CA, Meroz CL, Tang Y, McKenna KE, McVary KT. Regulation of cavernous nerve injury-induced apoptosis by sonic hedgehog. Biol Reprod 2007; **76**:19-28

8. Kovanecz I, Ferrini MG, Vernet D, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. Ageing-related corpora veno-occlusive dysfunction in the rat is ameliorated by pioglitazone. BJU Int 2007; **100**:867-74

9. Kovanecz I, Rambhatla A, Ferrini MG, Vernet D, Sanchez S, Rajfer J, Gonzalez-Cadavid NF. Tadalafil prevents the corporal veno-occlusive dysfunction (CVOD) that occurs following bilateral cavernosal nerve resection in the rat. BJU Int 2007 Sep 20; [Epub ahead of print]

10. Kovanecz I, Ferrini MG, Vernet D, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. Pioglitazone prevents corporal veno-occlusive dysfunction in a rat model of type 2 diabetes mellitus. BJU Int 2006; **98**:116-24

11: Jiang R, Chen JH, Jin J, Shen W, Li QM. Ultrastructural comparison of penile cavernous tissue between hypertensive and normotensive rats. Int J Impot Res 2005; **17**:417-23

12. Bakircioglu ME, Sievert KD, Nunes L, Lau A, Lin CS, Lue TF Decreased trabecular smooth muscle and caveolin-1 expression in the penile tissue of aged rats. J Urol 2001; **166**:734-8

13. User HM, Hairston JH, Zelner DJ, et al: Penile weight and cell subtype specific changes in a post-radical prostatectomy model of erectile dysfunction. J Urol 2003; **169**:1175-9

14. Leungwattanakij S, Bivalacqua TJ, Usta MF, et al: Cavernous neurotomy causes hypoxia and fibrosis in rat corpus cavernosum. J. Androl 2003; **24**:239-249

15. Kovanecz I, Rambhatla A, Ferrini MG, Vernet D, Sanchez S, Rajfer J, Gonzalez-Cadavid NF. Long term continuous sildenafil treatment ameliorates corporal veno-occlusive dysfunction

(CVOD) induced by cavernosal nerve resection in rats. Int J Impot Res 2007 Sep 20; [Epub ahead of print]

16. Ferrini MG, Davila HH, Kovanecz I, Sanchez SP, Gonzalez-Cadavid NF, Rajfer J. Vardenafil prevents fibrosis and loss of corporal smooth muscle that occurs after bilateral cavernosal nerve resection in the rat. Urology 2006; **68**:429-35

17. Melman A.Gene transfer for the therapy of erectile dysfunction: progress in the 21st century. Int J Impot Res 2006; **18**:19-25

18. Mimeault M, Hauke R, Batra SK. Stem cells: a revolution in therapeutics-recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. Clin Pharmacol Ther 2007; **82**:252-64

19. Becker C, Jakse G. Stem cells for regeneration of urological structures. Eur Urol 2007; **51**:1217-28

20. Bochinski D, Lin GT, Nunes L, Carrion R, Rahman N, Lin CS, Lue TF. The effect of neural embryonic stem cell therapy in a rat model of cavernosal nerve injury. BJU Int 2004; **94**:904-9

21. Song YS, Lee HJ, Park IH, Kim WK, Ku JH, Kim SU. Potential differentiation of human mesenchymal stem cell transplanted in rat corpus cavernosum toward endothelial or smooth muscle cells. In J Impot Res 2007; **19**:378-85

22: Bivalacqua TJ, Deng W, Kendirci M, Usta MF, Robinson C, Taylor BK, Murthy SN, Champion HC, Hellstrom WJ, Kadowitz PJ. Mesenchymal stem cells alone or ex vivo gene modified with endothelial nitric oxide synthase reverse age-associated erectile dysfunction. Am J Physiol Heart Circ Physiol 2007; **292**:H1278-90

23. Kim Y, de Miguel F, Usiene I, Kwon D, Yoshimura N, Huard J, Chancellor MB. Injection of skeletal muscle-derived cells into the penis improves erectile function. Int J Impot Res 2006; **18**:329-34

24: Mitterberger M, Pinggera GM, Marksteiner R, Margreiter E, Fussenegger M, Frauscher F, Ulmer H, Hering S, Bartsch G, Strasser H. Adult Stem Cell Therapy of Female Stress Urinary Incontinence. Eur Urol 2007 Jul 23; [EPub]

25: Frimberger D, Lin HK, Kropp BP. The use of tissue engineering and stem cells in bladder regeneration. Regen Med 2006; **1**:425-35

26: Rodriguez LV, Alfonso Z, Zhang R, Leung J, Wu B, Ignarro LJ. Clonogenic multipotent stem cells in human adipose tissue differentiate into functional smooth muscle cells. Proc Natl Acad Sci U S A 2006; **103**:12167-72

27. Rastogi A, Nissenson AR. The future of renal replacement therapy. Adv Chronic Kidney Dis 2007; **14**:249-55

28. Urish K, Kanda Y, Huard J. Initial failure in myoblast transplantation therapy has led the way toward the isolation of muscle stem cells: potential for tissue regeneration. Curr Top Dev Biol 2005; **68**:263-80

29. Engelmann MG, Franz WM. Stem cell therapy after myocardial infarction: ready for clinical application? Curr Opin Mol Ther 2006; **8**:396-414

30. Hwang JH, Yuk SH, Lee JH, Lyoo WS, Ghil SH, Lee SS, Khang IG, Paik SY, Lee JY.Isolation of muscle derived stem cells from rat and its smooth muscle differentiation [corrected]. Mol Cells. 2004; **17**:57-61. Erratum in: Mol Cells 2004; **17**:381

31. Kwon D, Kim Y, Pruchnic R, Jankowski R, Usiene I, de Miguel F, Huard J, Chancellor MB. Periurethral cellular injection: comparison of muscle-derived progenitor cells and fibroblasts with regard to efficacy and tissue contractility in an animal model of stress urinary incontinence. Urology 2006; **68**:449-54

32. Vernet D, Qian A, Nolazco G, Cantini L, Magee TR, Ferrini MG, Rajfer J, Gonzalez-Cadavid NF Evidence that osteogenic progenitor cells in the human tunica albuginea may originate from stem cells. Implications for Peyronie's disease. Biol Reprod 2005; **73**:1199-1210

33. Qu-Petersen Z, Deasy B, Jankowski R, Ikezawa M, Cummins J, Pruchnic R,Mytinger J, Cao B, Gates C, Wernig A, Huard J Identification of a novel population of muscle stem cells in mice: potentialfor muscle regeneration. J Cell Biol 2002; **157**:851-864

34. Blank, R.S., McQuinn, T.C., Yin, K.C., Thompson, M.M., Takeyasu, K., Schwartz, R.J. and Owens, G.K. Elements of the smooth muscle alpha-actin promoter required in cis for transcriptional activation in smooth muscle. Evidence for cell type-specific regulation. J. Biol. Chem 1992; **267**: 984-9

35. Ma W, Rogers K, Zbar B, Schmidt L. Effects of different fixatives on beta-galactosidase activity. J Histochem Cytochem 2002; **50**:1421-4

36. Magee TR, Kovanecz I, Davila HH, Ferrini MG, Cantini L, Vernet D, Zuniga FI, Rajfer J, Gonzalez-Cadavid NF.Antisense and short hairpin RNA (shRNA) constructs targeting PIN (Protein Inhibitor of NOS) ameliorate aging-related erectile dysfunction in the rat. J Sex Med 2007; **4**:633-43

37. Magee TR, Ferrini M, Garban HJ, Vernet D, Mitani K, Rajfer J, Gonzalez-Cadavid NF. Gene therapy of erectile dysfunction in the rat with penile neuronal nitric oxide synthase. Biol Reprod. 2002; **67**:1033-41

38. Ross JJ, Hong Z, Willenbring B, Zeng L, Isenberg B, Lee EH, Reyes M, Keirstead SA, Weir EK, Tranquillo RT, Verfaillie CM. Cytokine-induced differentiation of multipotent adult progenitor cells into functional smooth muscle cells. J Clin Invest 2006; **116**:3139-49

39. Sinha S, Hoofnagle MH, Kingston PA, McCanna ME, Owens GK. Transforming growth factor-beta1 signaling contributes to development of smooth muscle cells from embryonic stem cells. Am J Physiol Cell Physiol 2004; **287**:C1560-8

40. Garban H, Vernet D, Freedman A, Rajfer J, Gonzalez-Cadavid NF Effect of aging on nitric oxide-mediated penile erection in the rat. Amer J Physiol 1995; **268**:H467-H475

41: Garban H, Marquez D, Magee T, Moody J, Rajavashisth T, Rodriguez JA, Hung A, Vernet D, Rajfer J, Gonzalez-Cadavid NF. Cloning of rat and human inducible penile nitric oxide synthase. Application for gene therapy of erectile dysfunction. Biol Reprod 1997; **56**:954-63

42: Moody JA, Vernet D, Laidlaw S, Rajfer J, Gonzalez-Cadavid NF. Effects of long-term oral administration of L-arginine on the rat erectile response. J Urol 1997; **158**:942-7

43. Romagnani P, Lasagni L, Mazzinghi B, Lazzeri E, Romagnani S. Pharmacological modulation of stem cell function. Curr Med Chem 2007; **14**:1129-39

44. Madhusoodanan KS, Murad F. NO-cGMP signaling and regenerative medicine involving stem cells. Neurochem Res 2007; **32**:681-94

45. Wang L, Gang Zhang Z, Lan Zhang R, Chopp M. Activation of the PI3-K/Akt pathway mediates cGMP enhanced-neurogenesis in the adult progenitor cells derived from the subventricular zone. J Cereb Blood Flow Metab 2005; **25**:1150-8

46. Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. The myofibroblast: one function, multiple origins. Am J Pathol 2007; **170**:1807-16

47. Darby IA, Hewitson TD. Fibroblast differentiation in wound healing and fibrosis. Int Rev Cytol 2007; **257**:143-79

48: Carrier S, Nagaraju P, Morgan DM, Baba K, Nunes L, Lue TF. Age decreases nitric oxide synthase-containing nerve fibers in the rat penis. J Urol 1997; **157**:1088-92

49: Musicki B, Kramer MF, Becker RE, Burnett AL. Age-related changes in phosphorylation of endothelial nitric oxide synthase in the rat penis. J Sex Med. 2005; **2**:347-55; discussion 355-7.

50: Hsieh PS, Bochinski DJ, Lin GT, Nunes L, Lin CS, Lue TF. The effect of vascular endothelial growth factor and brain-derived neurotrophic factor on cavernosal nerve regeneration in a nerve-crush rat model. BJU Int 2003; 92:470-5

51: Martin KA, Merenick BL, Ding M, Betalvero KM, Rzucidlo EM, Kozul CD, Brown DJ, Chiu HY, Shyu M, Drapeau BL, Wagner RJ, Powell RJ. Rapamycin promotes vascular smooth

muscle cell differentiation through IRS-1/PI 3 Kinase/Akt2 feedback signaling. J Biol Chem 2007; Sept 30 (Epub ahead of print)

LEGENDS TO FIGURES

Fig. 1. MDSC incubated in vitro generated myotubes, myofibroblasts, osteoblasts, and SMC. MDSC were incubated in myogenic medium for 3 weeks (A), fibrogenic medium for 2 weeks (B), osteogenic medium for 4 weeks (C), and regular medium at low serum/rapamycin for 4 weeks (D), and differentiation was assayed by immunocytochemistry for MHC-2 (A), αSMA (B), calponin (D), or histochemistry for alkaline phosphatase (AP) (C), on 6-well plates and transferred to 8-well removable chambers prior to staining. Quantitative image analysis was applied on B and C (Bar graphs). Magnification: A, D: 200 X; B, C: 400 X.

Fig. 2. MDSC implanted into the rat corpora cavernosa remained proliferating after 2 weeks of implantation. DAPI-labeled MDSC ($0.8x10^6$ cells) were injected into the corpora cavernosa of young adult rats, and frozen penile sections were stained with propidium iodide (top panels), or with biotinylated antibody for PCNA detecting with streptavidin-Texas red (bottom panels). Images were obtained with blue (A, D) and red (B, E) filters in a regular fluorescent microscope and merged (C, F). Magnification: top: 100X, bottom: 200X.

Fig. 3 Confocal microscopy confirmed the expression of SMC markers in DAPI-labeled MDSC implanted into the corpora cavernosa, and the subsequent increase of corporal SMC was revealed by western blot. Frozen sections adjacent to those examined in Fig. 2. were immunostained for αSMA (top micrographs) or smoothelin (bottom micrographs) and examined in optical sections of 0.5 um using a confocal microscopy. Magnification: 200X. Bottom: the remaining tissues from penises injected with MDSC and control penile tissue that was not implanted with cells were homogenized and subjected to western blot for αSMA and PCNA loading equal amounts of protein. Magnification: 200X

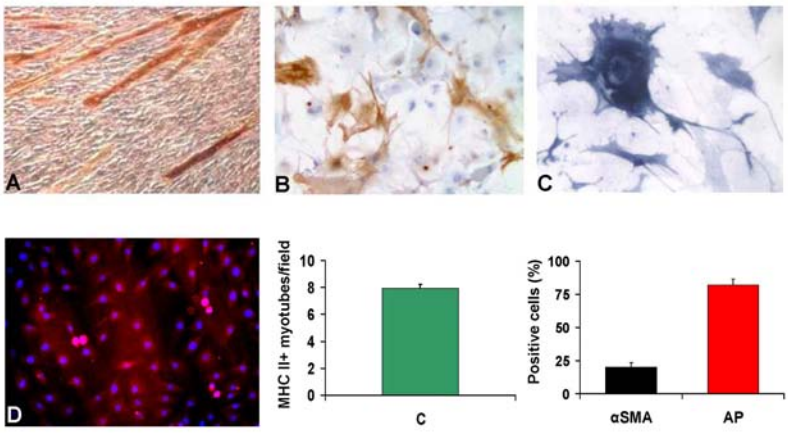
Fig. 4. MDSC were able to activate in vitro the αSMA promoter when stimulated with TGFβ1, or when injected in vivo into the corpora cavernosa. Rat corporal SMC (A) or mouse MDSC (B) were transfected in vitro with a plasmid expressing β-galactosidase under the αSMA promoter and were incubated for 7 days in the presence of TGFβ1 to induce αSMA expression. Wells were stained with X-gal and fast red. DAPI-labeled MDSC were also injected into the penile corpora cavernosa of adult rats, and the penile tissue was dissected. Frozen tissue sections obtained for examining DAPI nuclei (C) and then subjected to X-gal staining (D). Micrographs were obtained in the same section but not necessarily the same fields (no overlapping). Magnification: A, C: 100X, B: 400 X, D: 200X

Fig. 5. Stem cell marker CD34 was expressed endogenously in the tunica albuginea and corporal tissue non-implanted with MDSC. Top panels: Tissue sections from penises of young adult rats (not injected with MDSC) were stained for CD34 using either secondary antibody linked to peroxidase (top) or the fluorescent biotinylated secondary antibody and streptavidin Texas red (middle), and examined in the tunical and trabecular regions. Magnification: 400X. Bottom panel: western blot for CD34, conducted in penile tissue homogenates from the same rats, as well as from old rats.

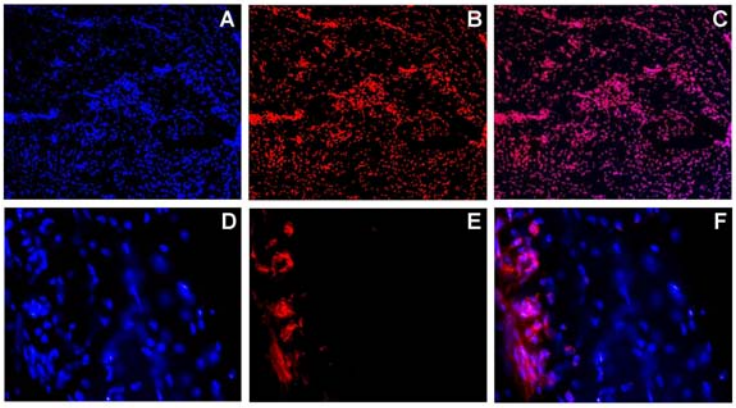
Fig. 6. Stem cell marker CD34 was expressed endogenously in the tunica albuginea and corporal tissue non-implanted with MDSC. Top panels: Tissue sections from penises of young adult rats not injected with MDSC were stained for Sca1 using either secondary antibody linked to peroxidase (top left) or the fluorescent biotinylated secondary antibody and streptavidin Texas red (top right), and examined in the tunical and trabecular regions. Magnification: 400X (left); 200X (right). Bottom panel: western blot for Sca1, conducted in penile tissue homogenates from the same rats, as well as from old rats. Sca1+: MDSC selected for Sca1 expression, low passage; NS: non-selected MDSC at high passage.

Fig. 7. MDSC implantation into the corpora cavernosa stimulated the erectile response to EFS in aged rats. 20 month old rats were injected with MDSC (B, C) or with saline as controls

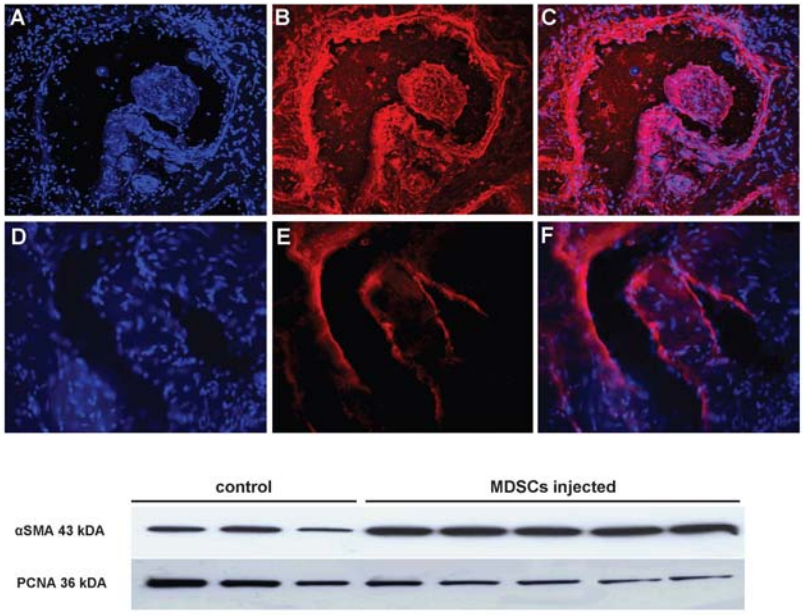
(A) and subjected to EFS after 2 (B) and 4 weeks (C). MIP: maximal intracavernosal pressure; MAP: mean arterial pressure. *P<0.05; **P<0.01.



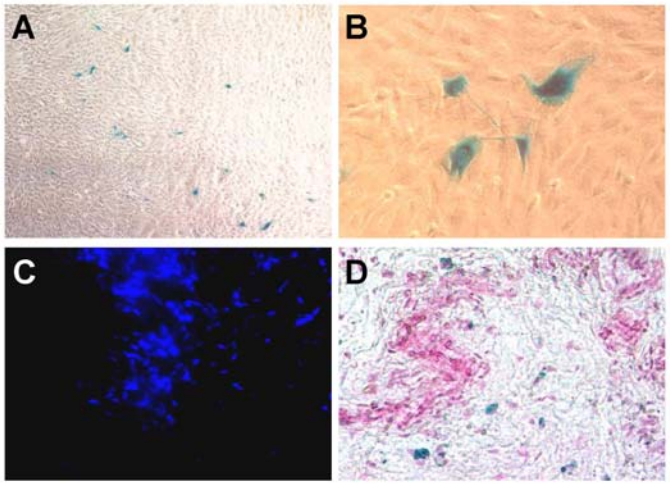
253x189mm (300 x 300 DPI)



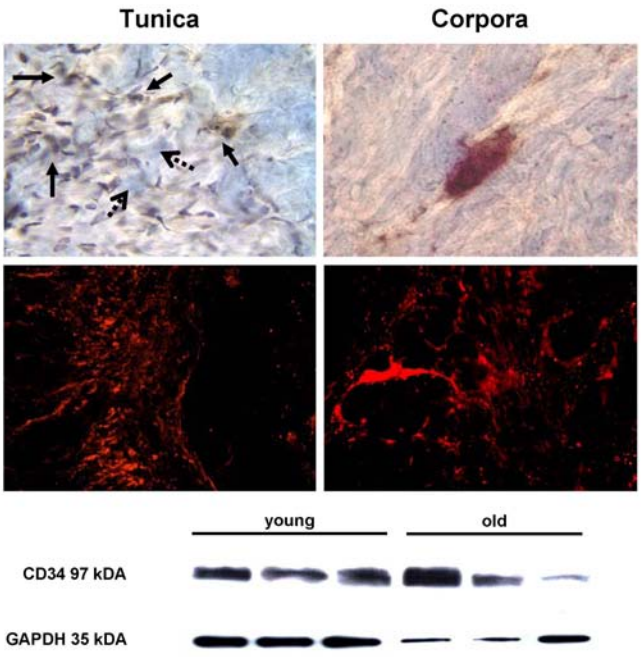
253x189mm (300 x 300 DPI)



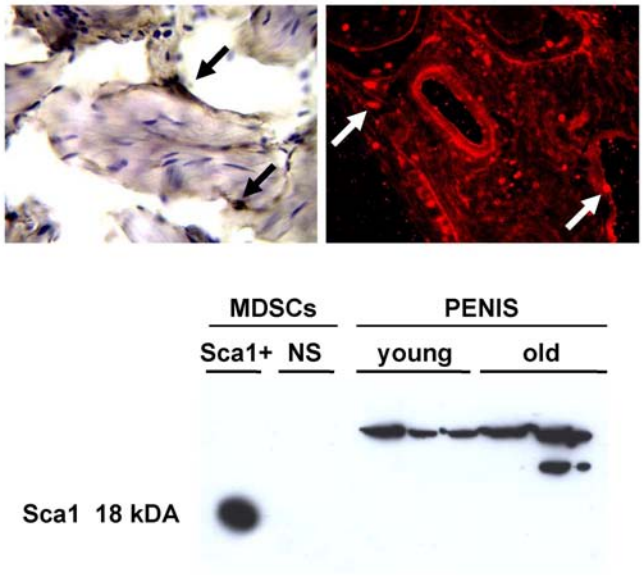
253x189mm (300 x 300 DPI)



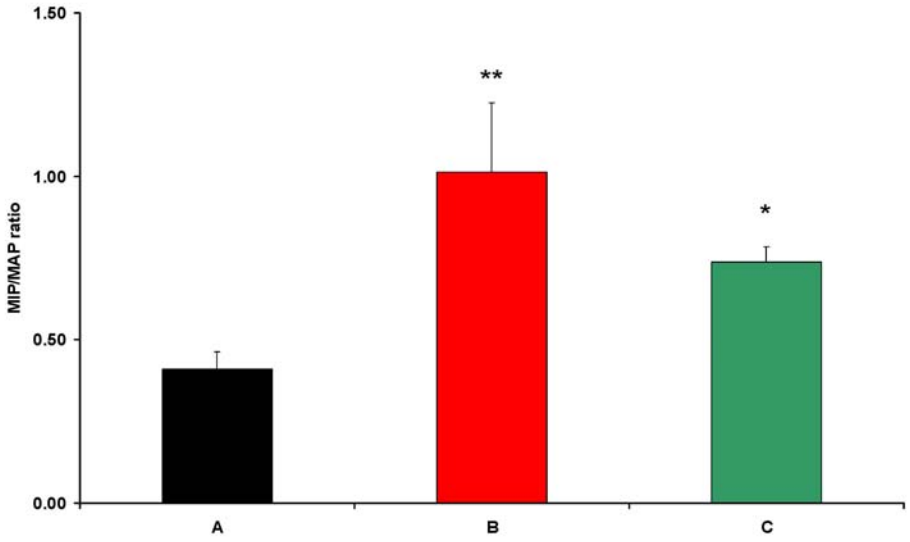
253x189mm (300 x 300 DPI)



253x189mm (300 x 300 DPI)



253x189mm (300 x 300 DPI)



253x189mm (300 x 300 DPI)

Myostatin promotes a fibrotic phenotypic switch in multipotent C3H 10T1/2 cells without affecting their differentiation into myofibroblasts

Jorge N Artaza[1,2], Rajan Singh[1], Monica G Ferrini[2,3], Melissa Braga[1], James Tsao[1] and Nestor F Gonzalez-Cadavid[1,3]

[1]Division of Endocrinology, Metabolism and Molecular Medicine and RCMI Molecular Core, [2]Department of Biomedical Sciences, The Charles R Drew University of Medicine and Science, 1731 East 120th Street, Los Angeles, California 90059, USA

[3]Department of Urology, UCLA David Geffen School of Medicine, Los Angeles, California 90095, USA

(Correspondence should be addressed to J N Artaza at the Division of Endocrinology, Metabolism and Molecular Medicine, Charles Drew University of Medicine and Science; Email: jorgeartaza@cdrewu.edu)

Abstract

Tissue fibrosis, the excessive deposition of collagen/extracellular matrix combined with the reduction of the cell compartment, defines fibroproliferative diseases, a major cause of death and a public health burden. Key cellular processes in fibrosis include the generation of myofibroblasts from progenitor cells, and the activation or switch of already differentiated cells to a fibrotic synthetic phenotype. Myostatin, a negative regulator of skeletal muscle mass, is postulated to be involved in muscle fibrosis. We have examined whether myostatin affects the differentiation of a multipotent mesenchymal mouse cell line into myofibroblasts, and/or modulates the fibrotic phenotype and Smad expression of the cell population. In addition, we investigated the role of follistatin in this process. Incubation of cells with recombinant myostatin protein did not affect the proportion of myofibroblasts in the culture, but significantly upregulated the expression of fibrotic markers such as collagen and the key profibrotic factors transforming growth factor-β1 (TGF-β1) and plasminogen activator inhibitor (PAI-1), as well as Smad3 and 4, and the pSmad2/3. An antifibrotic process evidenced by the upregulation of follistatin, Smad7, and matrix metalloproteinase 8 accompanied these changes. Follistatin inhibited TGF-β1 induction by myostatin. Transfection with a cDNA expressing myostatin upregulated PAI-1, whereas an shRNA against myostatin blocked this effect. In conclusion, myostatin induced a fibrotic phenotype without significantly affecting differentiation into myofibroblasts. The concurrent endogenous antifibrotic reaction confirms the view that phenotypic switches in multipotent and differentiated cells may affect the progress or reversion of fibrosis, and that myostatin pharmacological inactivation may be a novel therapeutic target against fibrosis.

Journal of Endocrinology (2008) **196,** 235–249

Introduction

Progressive scarring (fibrosis) is the main pathological process in fibroproliferative diseases. During advanced stages, these disorders are responsible for close to 45% of all deaths in the developed world (Wynn 2007). They often involve a relative loss of cells essential to normal tissue function. Fibrosis is analogous to abnormal wound healing occurring during tissue response due to chronic and sustained injury, microtrauma, oxidative stress, endogenous or exogenous insults, autoimmunity, and other factors. This process affects multiple organs in localized, multifocal, or disseminated forms, in conditions such as systemic sclerosis, liver cirrhosis, progressive kidney disease, cardiovascular disease, pulmonary fibroses, macular degeneration, and muscle dystrophies (Willis *et al.* 2006, Gharaee-Kermani *et al.* 2007, Henderson & Iredale 2007). Although chronic inflammation usually precedes fibrosis, it is neither necessary nor sufficient to trigger it, and as a result anti-inflammatory agents are usually not effective against fibrosis. In fact, successful antifibrotic treatments are very rare (Wynn 2007).

At the cellular level, one of the main factors of fibrosis is the differentiation of a not well-defined progenitor (local mesenchymal stem cells; fibroblasts; or epithelial, smooth muscle, or stellate cells; or recruited exogenous cells such as perycites or bone marrow fibrocytes) into myofibroblasts, the cells that share a fibroblast/smooth muscle phenotype (Kisseleva & Brenner 2006, Qi *et al.* 2006, Iredale 2007). In fact, myofibroblasts are usually absent from normal tissue. They accumulate after injury or the impact of a noxious factor, and synthesize collagen and extracellular matrix during tissue repair. They then normally disappear by apoptosis when the process is completed. An increase in the differentiation of fibroblasts from their progenitors, or the failure of myofibroblasts to be removed after sufficient collagen has been deposited, is probably the basis of many fibroses (Iredale 2007, Wynn 2007). However, the activation of already differentiated contractile cells – myofibroblasts,

fibroblasts, or smooth muscle cells – to a synthetic phenotype where collagen is produced intensively is also a key step in fibrosis progression. Counteracting this myofibroblast differentiation and/or activation is therefore the primary target of novel therapies, considering that tissue defense mechanisms against these processes may operate in the now accepted concept of spontaneous reversibility of fibrosis, as in cirrhosis of the liver and kidney (Iredale 2007, Wynn 2007).

Many members of the transforming growth factor-β (TGF-β) superfamily, particularly TGF-β1 and activin A, are well-known profibrotic factors that play a role in most of the stages in this process in many organs. This occurs specifically in the stimulation of myofibroblast differentiation, the switch to the synthetic phenotype, and in the inhibition of apoptosis (Sulyok *et al.* 2004, Mauviel 2005, Ruiz-Ortega *et al.* 2007). They upregulate collagen synthesis, the expression of ancillary profibrotic proteins, and of each other, as in the case of PAI-1 or connective tissue growth factor. They also share the downstream Smad signaling pathway.

Smad3 is a well-known profibrotic agent, more than Smad2, whereas Smad6 and 7 are antifibrotic, similar to another upstream member of the TGF-β1 family, bone morphogenic protein 7 (BMP-7; Wang *et al.* 2005, Ask *et al.* 2006, Liu 2006). Moreover, follistatin, a protein that binds and inactivates TGF-β1 and activin A, and downregulates the expression of their mRNAs, is antifibrotic in a number of conditions, namely, lung, liver, and kidney fibrosis (Aoki *et al.* 2005, Patella *et al.* 2006).

Myostatin, the only known negative regulator of skeletal muscle mass, is also a member of the TGF-β superfamily (Lee 2004, Tsuchida 2004). It has been shown to modulate multipotent cell differentiation, specifically in the C3H10T1/2 mouse embryonic cell line of fibroblast origin, where myostatin stimulates adipogenic commitment while inhibiting myogenic lineage, whereas androgens exert the opposite effect (Singh *et al.* 2003, Artaza *et al.* 2005, Jasuja *et al.* 2005, Feldman *et al.* 2006). C3H10T1/2 is a well-known and widely used multipotent mesenchymal cell line that undergoes differentiation into several cell lineages (Taylor & Jones 1979, Atkinson *et al.* 1997, Fischer *et al.* 2002, Wilson & Rotwein 2006).

In addition, myostatin inactivation increases osteogenic differentiation in bone marrow stem cells (Hamrick *et al.* 2007). Recent indirect evidence suggests that myostatin acts in the same way as other members of the TGF-β family, by inducing fibrosis in the skeletal muscle (Engvall & Wewer 2003, McCroskery *et al.* 2005). This is likely to occur via the Smad pathway, since myostatin signals through Smad2–4, and phosphorylates Smad3 (Zhu *et al.* 2004). In addition, it triggers a feedback compensatory mechanism through the inhibitory Smad7 that inactivates myostatin promoter, and counteracts myostatin and TGF-β action (Zhu *et al.* 2004, Forbes *et al.* 2006, Kollias *et al.* 2006). In turn, Smad2–4 upregulate myostatin expression by activating its promoter (Zhu *et al.* 2004).

Similar to TGF-β1 and activin A, myostatin is inhibited by follistatin, and also by the agents that induce follistatin expression, such as deacetylase inhibitors that block myostatin negative action on the muscle (Lee & McPherron 2001, Amthor *et al.* 2004, Iezzi *et al.* 2004, Kocamis *et al.* 2004). However, it is not known whether myostatin's putative profibrotic action is exerted via the stimulation of myofibroblasts differentiation from their progenitor, or by inducing a switch of the differentiated cell to the synthetic phenotype producing extracellular matrix. It is also unclear whether follistatin neutralizes these effects.

In our current work, we examined the effects of recombinant and endogenous myostatin on a) the differentiation of the multipotent C3H10T1/2 cell line into myofibroblasts, b) the regulation of the expression of TGF-β1 and other fibrotic-related genes, and c) proteins involved in the Smad signaling cascade and follistatin. In addition, we investigated whether a) follistatin counteracts the effect of myostatin, b) over-expression of myostatin mRNA mimics the paracrine effects of the exogenous protein, and c) this effect is blocked by the myostatin shRNA.

Materials and Methods

Cell culture

Mouse C3H10T1/2 multipotent cells (ATCC, Manassas, VA, USA) grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum at 37 °C were treated with or without 20 μM 5′-azacytidine (AZCT) for 3 days to induce differentiation (Singh *et al.* 2003, Artaza *et al.* 2005). Cells were split in a 1:2 ratio; allowed to recover for 2 days; seeded onto 60–70% confluence in T75 flasks, eight-well chamber slides or six-well plates; and incubated with 4 μg/ml recombinant 113 amino acid myostatin protein (R-Mst; Artaza *et al.* 2005) in DMEM–10% serum for 0·5, 1, 2, 3, and 24 h and for 3, 4, and 10 days to assess paracrine effects of myostatin (see the section below). Azacytidine-treated cells were also co-transfected at 60% confluence in six-well plates with a) a myostatin cDNA plasmid expressing the full-length 375 amino acid protein (pcDNA-Mst-375; Taylor *et al.* 2001) or b) the silencer RNAs for two different myostatin sequences (pSil-Mstno.4 and 326) (Artaza *et al.* 2005, Magee *et al.* 2006) (see the section below). A pcDNA3.1-EGFP (Invitrogen) was co-transfected separately to evaluate the transfection efficiency.

siRNA myostatin

siRNA myostatin was described by Artaza *et al.* (2005) and Magee *et al.* (2006). To prepare the silencer RNA construct for myostatin, we analyzed the mouse myostatin gene sequence (accession no. NM_010834) using the web-based siRNA target finder and design tool provided in the Ambion website (Ambion Inc., Austin, TX, USA). Five regions were initially targeted for likely inhibitory activity by siRNAs (nucleotide position target no.): 176(no. 4), 207(no. 8), 426(no. 26), 647(no. 45), and

1064(no. 72)). Double-stranded RNAs were transcribed *in vitro* using the Silencer siRNA Construction Kit. In addition, a control siRNA targeting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and provided with the kit was also synthesized. Each siRNA was tested for inhibitory activity at 1, 10, and 100 nM concentrations by co-transfection of pCDNA3.1-Mst into human embryonic kidney cells (HEK)293 cell cultures using Lipofectamine 2000 (Invitrogen). After 48 h, cell lysates were collected in M-PER (Pierce Biotechnology Inc., Rockford, IL, USA) and western blot analyses were performed. Two siRNAs (growth differentiation factor (GDF)8 siRNA26 and siRNA4) were found to inhibit more than 95% of the myostatin gene expression (Magee *et al.* 2006).

Based on a GenBank Blast search, these sequences have homology not only to mouse but also to human, rat, rabbit, cow, macaque, and baboon. A short hairpin DNA sequence was synthesized and cloned into the pSilencer 2.1-U6 neo (Ambion Inc.), according to the manufacturer's instructions. The DNA sequence consists of a *Bam*HI DNA restriction site, sense strand, nine-nucleotide loop, antisense strand, RNA polymerase III terminator, and *Hin*dIII DNA restriction site 5′ to 3′ (Artaza *et al.* 2005, Magee *et al.* 2006). The pSilencer 2.1-U6 neo-GDF8 siRNA plasmid constructs were transfected (1 μg/well plate, six-well plate) into C3H10T1/2 cell cultures using Lipofectamine 2000 following the manufacturer's instructions as before.

Qualitative and quantitative immunocytochemical analyses

Cells grown in eight-well chamber slides were fixed in 2% *p*-formaldehyde, quenched with H_2O_2, blocked with normal goat or horse serum and incubated with specific antibodies (Artaza *et al.* 2002, Singh *et al.* 2003, 2006, Artaza *et al.* 2005, Jasuja *et al.* 2005). These consisted of a α-smooth muscle actin immunohistology kit (Sigma–Aldrich), and antibodies against pSmad2/3 (1:500); Smad3 (1:500), Smad4 (1:500) and Smad7 (1:500) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); PAI-1 (1:200; Abcam Inc.); TGF-β1 (1:200) (Promega Corporation); collagen I (1:100) and collagen III (1:100; Chemicon International Inc., Temecula, CA, USA).

Detection was based on a secondary biotinylated antibody (1:200), followed by the addition of the streptavidin–horseradish peroxidase ABC complex (1:100), Vectastain (Elite ABC System, Vector Laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine and H_2O_2 mixture (Sigma). The cells were counterstained with Mayer's hematoxylin solution (Sigma). In negative controls, we either omitted the first antibody or used a rabbit non-specific IgG.

In all cases, the cytochemical staining was quantitated by image analysis using ImagePro-Plus 5.1 software (Media Cybernetics, Silver Spring, MD, USA) coupled to a Leica digital microscope bright field light fluorescence microscope/VCC video camera. After images were calibrated for background lighting, wherein integrated OD (IOD = area × average intensity) was calculated using at least ten pictures per treatment group per well done in duplicate. These experiments were repeated at least thrice (Singh *et al.* 2003, Artaza *et al.* 2005, Jasuja *et al.* 2005).

Western blot and densitometry analyses

Cell lysates (40–80 μg protein) were subjected to western blot analyses (Artaza *et al.* 2002, Singh *et al.* 2003) by 4–15% Tris–HCl PAGE (Bio-Rad) in running buffer (Tris/glycine/SDS). Proteins were transferred overnight at 4 °C to nitrocellulose membranes in transfer buffer (Tris/glycine/methanol). The next day the non-specific binding was blocked by immersing the membranes in 5% non-fat dried milk and 0·1% (v/v) Tween 20 in PBS for 1 h at room temperature. After several washes with washing buffer (PBS Tween 0·1%), the membranes were incubated with the primary antibodies for 1 h at room temperature. Monoclonal antibodies were as follows: a) α-smooth muscle actin (1:1000; Calbiochem, La Jolla, CA, USA) and b) GAPDH; 1:10 000) (Chemicon International). Polyclonal antibodies were used for a) Smad3 (1:200), b) Smad7 (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), c) TGF-β1 (1:1000; Promega Corporation), and d) myostatin (1:1000) (Chemicon International Inc). The washed membranes were incubated for 1 h at room temperature with 1:3000 dilution (anti-mouse) or 1:2000 dilution (anti-rabbit) of secondary antibody linked to horseradish peroxidase respectively. After several washes, the immunoreactive bands were visualized using the emission of chemiluminescence (ECL) plus Western blotting chemiluminescence detection system (Amersham Biosciences). The densitometry analysis of the bands was done with the Scion Image software beta 4.0.2 (Scion Corp., Frederick, MD, USA).

Recombinant proteins

Myostatin recombinant protein was produced in *Escherichia coli* as a 16 kDa protein containing 113 amino acid residues of the human myostatin protein (BioVendor Laboratory Medicine Inc., Palackeho, Czech Republic). Recombinant follistatin protein was generated in Sf21 cells as a 31 kDa protein containing 289 amino acid residues (R&D Systems, Minneapolis, MN, USA). Recombinant human TGF-β1 was produced in Chinese hamster ovary (CHO) cells as a 25 kDa protein containing 112 amino acid residues (Chemicon International Inc).

Real-time quantitative PCR

Total RNA was extracted using Trizol reagent (Invitrogen) and equal amounts (2 μg) of RNA were reverse transcribed using a RNA PCR kit (Applied Biosystems, Foster City, CA, USA). The locations of forward/reverse PCR primers for real-time RT-PCR are as follows: Smad7 region 575–597/626–641 on BC074818.2 (67 bp); follistatin (Fst; 150 bp), PPM04451A on NM_008046 and GAPDH (152 bp), 606–626/758–738 on BC023196. Mouse gene PCR primer sets (RT2) were purchased from SuperArray Bioscience (Frederick, MD, USA). The Qiagenn Sybr Green

PCR kit with HotStar *Taq* DNA polymerase (Qiagen) was used with i-Cycler PCR thermocycler and fluorescent detector lid (Bio-Rad; Singh *et al.* 2006).

The protocol included melting for 15 min at 95 °C, 40 cycles of three-step PCR including melting for 15 s at 95 °C, annealing for 30 s at 58 °C, elongation for 30 s at 72 °C, with an additional detection step of 15 s at 81 °C, followed by a melting curve from 55 to 95 °C at the rate of 0·5 °C per 10 s. Fst annealing for primers, however, was at 55 °C and detection at 76 °C. We confirmed that inverse derivatives of melting curves show sharp peaks for Smad7 at 82 °C, Fst at 84·5 °C, and GAPDH at 87 °C, indicating the correct products. Samples of 25 ng cDNA were analyzed in quadruplicate in parallel with GAPDH controls; standard curves (threshold cycle versus log pg cDNA) were generated by log dilutions of from 0·1 pg to 100 ng standard cDNA (reverse-transcribed mRNA from C3H10T1/2 cells in AM). Experimental mRNA starting quantities were then calculated from the standard curves and averaged using i-Cycler, iQ software, as described previously (Singh *et al.* 2003). The ratios of marker experimental gene (e.g. follistatin mRNA) to GAPDH mRNA were computed and normalized to control (untreated) samples as 100%.

DNA microarray analysis of TGF-β/BMP target genes

Pools of total cellular RNA from three different T75 flasks for each experimental condition were isolated with Trizol reagent from C3H10T1/2 cells undergoing differentiation with AZCT and treated with or without recombinant myostatin protein (4 μg/ml) for 3 h and 3 days. Isolated RNA was subjected to cDNA gene microarrays (GEArray Q Series, TGF-β/BMP signaling pathway gene array (matrix microarray (MM)-023) and osteogenesis gene array (MM-026) analysis (SuperArray BioScience Corp). The microarray assays were performed in two separate experiments in each case. This series of mice TGF-β/BMP signaling pathway gene arrays are designed to study the genes involved in TGF-β/BMP signaling pathway (MM-023) and the osteogenic array (MM-026) contains collagens of some other fibrotic-related genes. Biotin-labeled cDNA probes were synthesized from total RNA, denatured, and hybridized overnight at 60 °C in GEHybridization solution to membranes spotted with TGF-β/BMP signaling pathway-specific genes, as well as with genes involved in the regulation of osteogenic differentiation. Membranes were then washed, and chemi-luminescent analysis was performed as per the manufacturer's instructions. Raw data were analyzed using GEArray Expression Analysis Suite (SuperArray BioScience Corp). Fold changes in relative gene expression were presented after background correction and normalization with a housekeeping gene.

Confirmation of DNA microarray analysis by RT^2 profiler PCR array analysis of TGF-β/BMP target gene

RT^2 profiler PCR SuperArray analyses of TGF-β/BMP target genes were applied in order to confirm selected genes from the GEArray data. Aliquots of total cellular RNA isolated with Trizol reagent from C3H10T1/2 cells undergoing differentiation with AZCT were treated with or without recombinant myostatin protein (4 μg/ml) for 3 h and 3 days. They were then subjected to reverse transcription, and the resulting cDNA was analyzed by RT^2 profiler PCR mouse TGF-β/BMP signaling pathway (APM-035A) (SuperArray BioScience Corp). This series of mouse TGF-β/BMP signaling pathway gene array is designed to study the genes involved in and downstream of TGF-β/BMP signaling. Each array contains a panel of 84 primer sets related to the TGF-β/BMP signaling genes plus 5 housekeeping genes and two negative controls. Real-time PCRs were performed as follows: a) melting for 10 min at 95 °C, b) 40 cycles of two-step PCR including melting for 15 s at 95 °C, and c) annealing for 1 min at 60 °C. The raw data were analyzed using the $\Delta\Delta C_t$ method following the manufacturer's instructions (SuperArray).

Statistical analysis

All data are presented as mean ± S.E.M., and between-group differences were analyzed using ANOVA. If the overall ANOVA revealed significant differences, then pair-wise comparisons between groups were performed by Newman–Keuls multiple comparison test. All comparisons were two-tailed, and $P<0{\cdot}05$ were considered statistically significant. The *in vitro* experiments were repeated thrice, and data from representative experiments are shown. Specifically, the DNA microarrays tests were done twice and the results confirmed by qRT-PCR in triplicate.

Results

Myofibroblast generation from multipotent C3H10T1/2 cells occurs spontaneously, and is not affected by azacytidine or incubation with exogenous myostatin, but myostatin triggers a fibrotic phenotype associated with transcriptional regulation of fibrotic-related genes and the Smad cascade

C3H10T1/2 cells, treated or non-treated with AZCT, were tested for the presence of myofibroblasts (cells with a fibroblast/smooth muscle cell hybrid phenotype, which play a key role in fibrosis), and also to determine whether myostatin stimulates C3H10T1/2 commitment to this differentiation lineage. The cultures not treated with AZCT had some cells that stained positive for α-smooth muscle actin (ASMA), a marker that is common to myofibroblasts and smooth muscle cells (data not shown). They had the typical appearance of myofibroblasts with prominent actin filaments and lamellipodia. Treatment of these cultures with either AZCT alone or in combination with recombinant myostatin protein did not alter this morphology (data not shown), nor there was any apparent increase from these treatments in terms of myofibroblast number (~8% of the total cell population) as confirmed by quantitative image analysis. Western immunoblot analysis of the cell extracts for ASMA compared with GAPH expression agreed with the qualitative

and quantitative immunocytochemistry observations. In addition, calponin, a marker for smooth muscle cells, which is not expressed in myofibroblasts, was not detected in the extracts (data not shown). This indicates that the $ASMA^+$ cells in the C3H10T1/2 cultures are indeed myofibroblasts.

Although myostatin did not stimulate myofibroblast differentiation in the azacytidine-induced multipotent C3H10T1/2 culture, it switched these cells towards the synthetic fibrotic phenotype, as shown in Fig. 1. Starting with this experiment, cells were always treated with AZCT, even if this drug did not affect the number of myofibroblasts that originated from C3H10T1/2 cells. This was done in order to facilitate comparisons with studies, including our previous results (Singh *et al.* 2003, Artaza *et al.* 2005, Jasuja *et al.* 2005) where azacytidine was routinely employed to stimulate multipotent stem cell differentiation (Schmittwolf *et al.* 2005). Incubation with recombinant myostatin protein clearly stimulated the intensity of immunocytochemical staining for the four selected fibrotic markers PAI-1, TGF-β1, collagen I, and collagen III (Fig. 1A and B, left panels). Most of the cells had a very low basal level of expression in the absence of treatment, but the expression per cell was intensified by myostatin. This is reflected

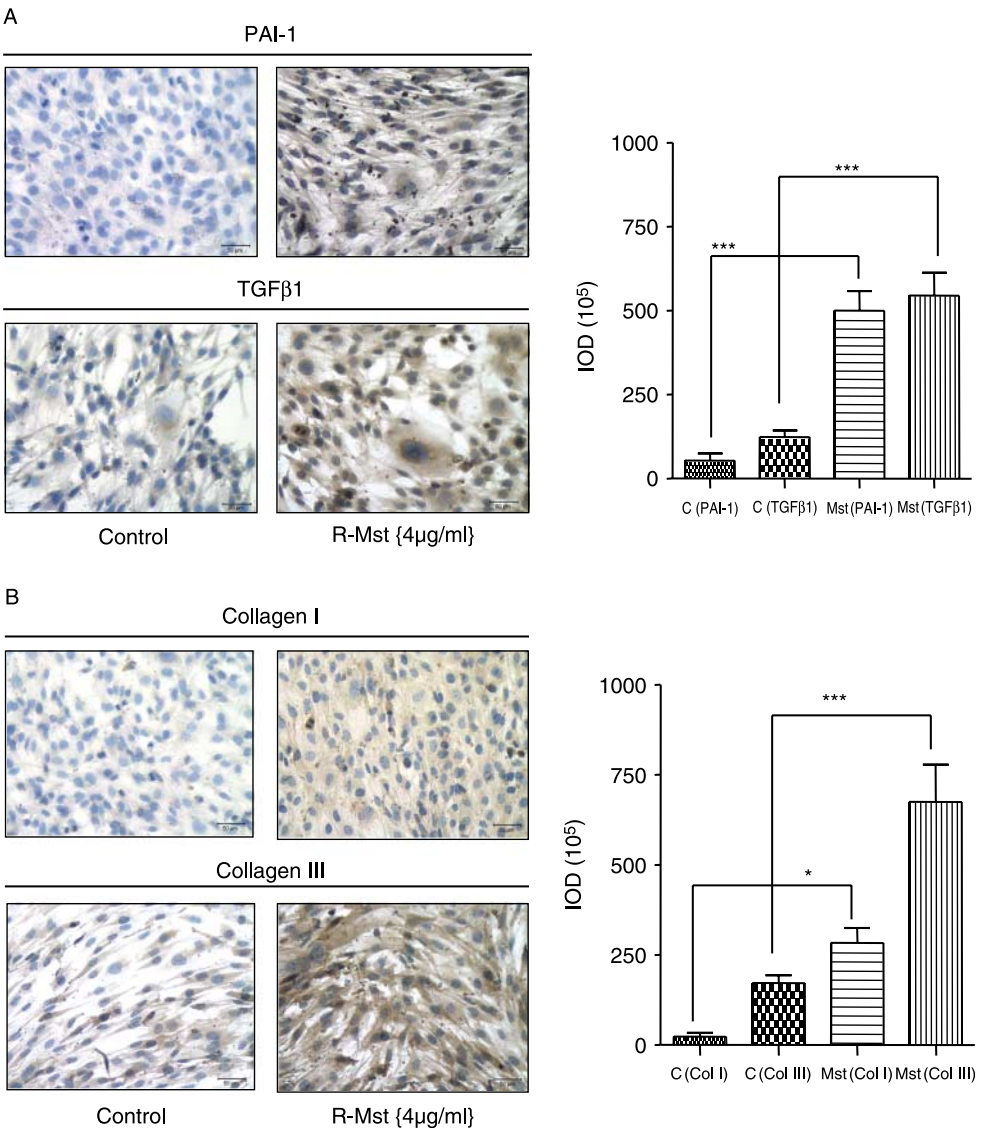


Figure 1 Effects of recombinant myostatin protein on the expression of profibrotic genes and collagen in C3H10T1/2 cells. Representative pictures (200×) of cells treated with azacytidine (20 μM) to induce differentiation and which, 2 days later, received myostatin (4 μg/ml), or no myostatin, on eight-well removable chambers, for 3 days followed by immunostaining (left) with the corresponding antibodies. (A) Profibrotic genes: PAI-1 and TGF-β1 and (B) collagen I and III. Mean ± S.E.M. corresponds to experiments done in triplicate of the integrated optical densities (IOD). $^*P<0{\cdot}05$, $^{***}P<0{\cdot}001$ (200×).

on the quantitative image analysis (Fig. 1A and B, right panels) that shows a statistically significant increase in PAI-1, TGF-β1, collagen I, and collagen III expression after incubation with recombinant myostatin (R-Mst) for 4 days.

The stimulation of fibrotic gene expression in the C3H10T1/2 cells by myostatin was also analyzed at the transcriptional level using mouse osteogenesis gene array (MM-026) and mouse TGF-β/BMP signaling pathway gene array (MM-023) (data not shown).

Figure 2 shows one of the two sets of membranes for DNA mouse osteogenesis gene array assay performed on total RNA extracted from cells subjected for 3 h (2A) and 3 days (2B) of incubation with or without recombinant myostatin. Some of the genes that showed differential RNA expression between the myostatin-treated and -untreated cells are indicated by circles (Fig. 2A and B) and were selected for the table shown in Fig. 2C, where the computer-generated ratios of spot intensities, normalized by housekeeping genes are tabulated for 3 days in the left column. This PCR microarray panel has only some of the fibrotic genes – essentially collagens, BMP members, and all the Smad genes – that transduce signals triggered by the members of the TGF-β1 family. For this reason, other genes selected from the mouse TGF-β/BMP panel are also included. The change of expression of some selected genes at 3 days was ultimately confirmed by real-time PCR using the RT^2 profiler PCR SuperArray set of primers and procedures. The ratios for triplicate determinations are shown on the right column. The agreement between the ratios obtained by DNA microarrays and RT-PCR is in general adequate and provides a reasonable assessment of up- and downregulation. From both the columns and the quantitative microarray data for 3 h (not shown), it was found that the stimulation of the mRNA expression of collagen I α, collagen IX α1, Smad3, 4, and 7, BMP 3, BMP 6, BMP7, and v cam occurred early, whereas it took longer to enhance the levels of the mRNAs for collagen I α1, collagen IV α4, collagen IV α6, and MMP8. The increase in Smad7 mRNA by myostatin remained considerable at 3 days, whereas that for Smad3 and 4 mRNAs was negligible.

The early transcriptional stimulation of Smad3 mRNA expression by myostatin was demonstrated at the protein level by immunocytochemistry, as shown in the time course depicted in Fig. 3A. TGF-β1, the main profibrotic factor and the member of the TGF-β superfamily that includes myostatin, signals through the Smad pathway, and was therefore used as positive control. There was an early and dramatic increase in the intensity of Smad3 staining (Fig. 3A) that peaked at 1 h, and was later downregulated reaching normal values at 24 h. The 3-h expression was confirmed by western blot analysis, which showed that myostatin was nearly as effective as TGF-β1 (Fig. 3B).

The time course of exogenous myostatin induction of Smad proteins shows an early expression and phosphorylation of the Smad2–4 genes followed by a later upregulation of the inhibitory Smad7

Smad3 was not the only Smad protein modulated by myostatin. C3H10T1/2 cells were incubated with recombinant myostatin; a very early stimulation of the phosphorylated Smad2/3 proteins was observed at 30 min, peaking at 1 h. This decayed at 2 h and normalized at 24 h (Fig. 4A). A similar process after 1 h occurred with the expression of Smad4 protein (Fig. 4B).

Consistent with the results shown in Fig. 2, Smad7 was expressed early. However, the immunostaining remained high even at 6 h (Fig. 5A). Western blot analysis confirmed the early Smad7 expression induced by myostatin. This was even higher than that induced by TGF-β1 (Fig. 5B). On the other hand, real-time RT-PCR showed that the stimulation of Smad7 mRNA expression by myostatin persisted even at 3 days, when the other Smad proteins had fallen to negligible levels (Fig. 5C).

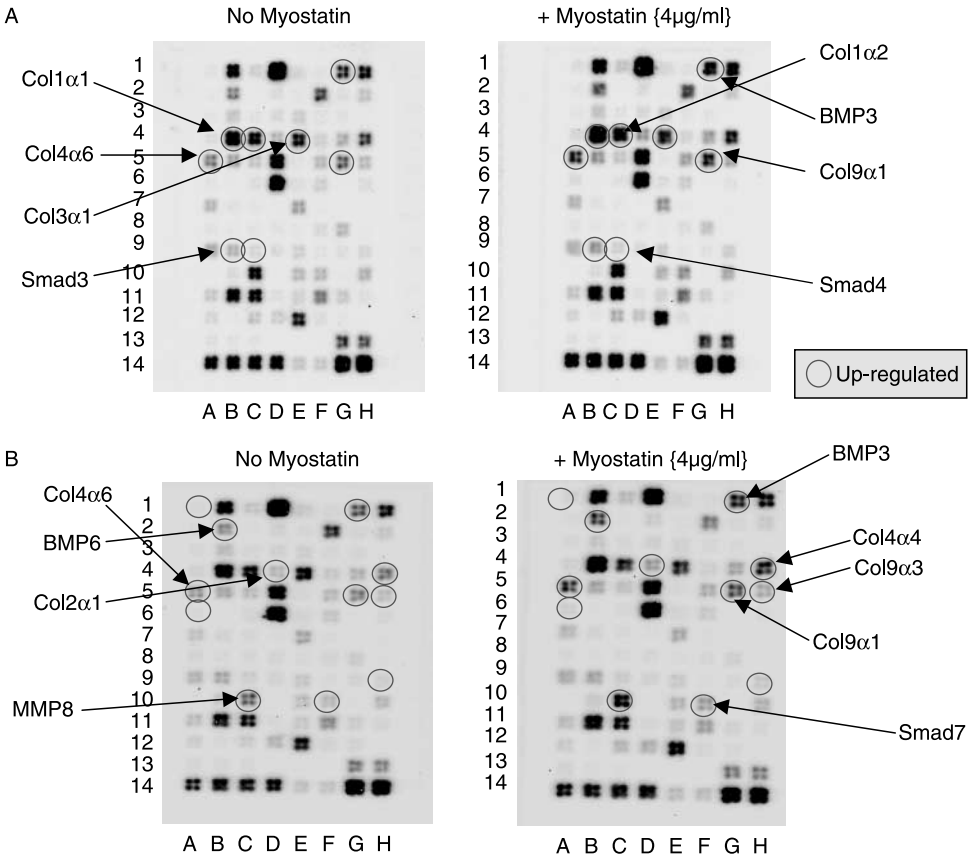
Exogenous myostatin upregulates the expression of its inhibitory protein, follistatin, and the addition of follistatin downregulates the myostatin-induced upregulation of TGF-β1

Since the activities of myostatin, TGF-β1, activin, and other members of the TGF-β family are inhibited by follistatin through binding to these proteins, we investigated whether myostatin modulated the expression of follistatin in the cells that were undergoing a fibrotic phenotypic differentiation. Contrary to our initial assumption that follistatin levels would be downregulated by myostatin, and would thus boost myostatin effects, real-time RT-PCR (Fig. 6A) revealed an early threefold stimulation at 3 h of follistatin mRNA levels that remained remarkably high even at 3 days.

To determine whether this was a compensatory mechanism of the C3H10T1/2 cells to counteract the profibrotic effects of myostatin, we tested whether the addition of recombinant follistatin protein would block the myostatin stimulation of the production of profibrotic factors by these cells. We also investigated whether myostatin modulation of the fibrotic phenotypic differentiation is mediated by the upregulation of the expression of TGF-β1, the main profibrotic factor. Figure 6B shows that at 4 days, myostatin stimulated TGF-β1 expression, and that the addition of follistatin (0·5 μg/ml) blocked this stimulation, whereas follistatin *per se* did not affect TGF-β1 expression. These effects were confirmed by western immunoblot (Fig. 6C).

The profibrotic effects of myostatin on C3H10T1/2 cells can also be exerted autocrinely by over-expression of myostatin mRNA, or through the breakdown of myostatin mRNA by its shRNA

The preceding experiments indicated that exogenous myostatin regulated the fibrotic phenotype of C3H10T1/2 cells which contain myofibroblasts that originated from this multipotent cell culture. Questions remained, however, whether endogenously produced myostatin would cause the same effects, and whether blocking its expression at the protein level would inhibit the production offibrotic factors. Figure 7 shows that transfection of these cells with plasmid constructs expressing myostatin increased myostatin expression, as evidenced by the western blot analysis (Fig. 7B, left) and the corresponding densitometry



C

Symbol	Description	Microarray Ratios (3 days)	Real time PCR Ratios (3 days)
Bmp3	Bone morphogenetic protein 3	2·8	3·5
Bmp6	Bone morphogenetic protein 6	2·7	3·6
Col1α1	Procollagen, type I, α1	2·8	3·3
Col1α2	Procollagen, type I, α2	2·9	3·5
Col3α1	Procollagen, type III, α1	2·8	3·1
Col2α1	Procollagen, type II, α1	2·7	3·9
Col4α4	Procollagen, type IV, α4	3·2	4·3
Col4α6	Procollagen, type IV, α6	3·1	3·9
Col9α1	Procollagen, type IX, α1	3·4	3·8
Col9α3	Procollagen, type IX,α3	3·2	3·8
Fst	Follistatin	2·4	2·9
Mmp8	Matrix metalloproteinase 8	3·1	3·9
Smad7	Mad homolog 7	2·0	2·5

Figure 2 Effects of recombinant myostatin protein on the transcriptional expression of Smad proteins, collagen, and other fibrotic genes in C3H10T1/2 cells. Cells were treated as shown in Fig. 1, but in 75 cm^2 flasks, for 3 h (A) and 3 days (B). Total RNA was isolated and subjected to DNA microarray analysis for genes related to extracellular matrix represented in the osteogenesis gene array. (A and B) Representative membranes of assays performed in two separate experiments. (C) In parallel reactions, total RNA was subjected to RT-real-time PCR by the RT^2-PCR profiler TGF-β array and the ratio between the myostatin-treated versus myostatin-untreated cells corrected by GAPDH was calculated for the assays performed in triplicate.

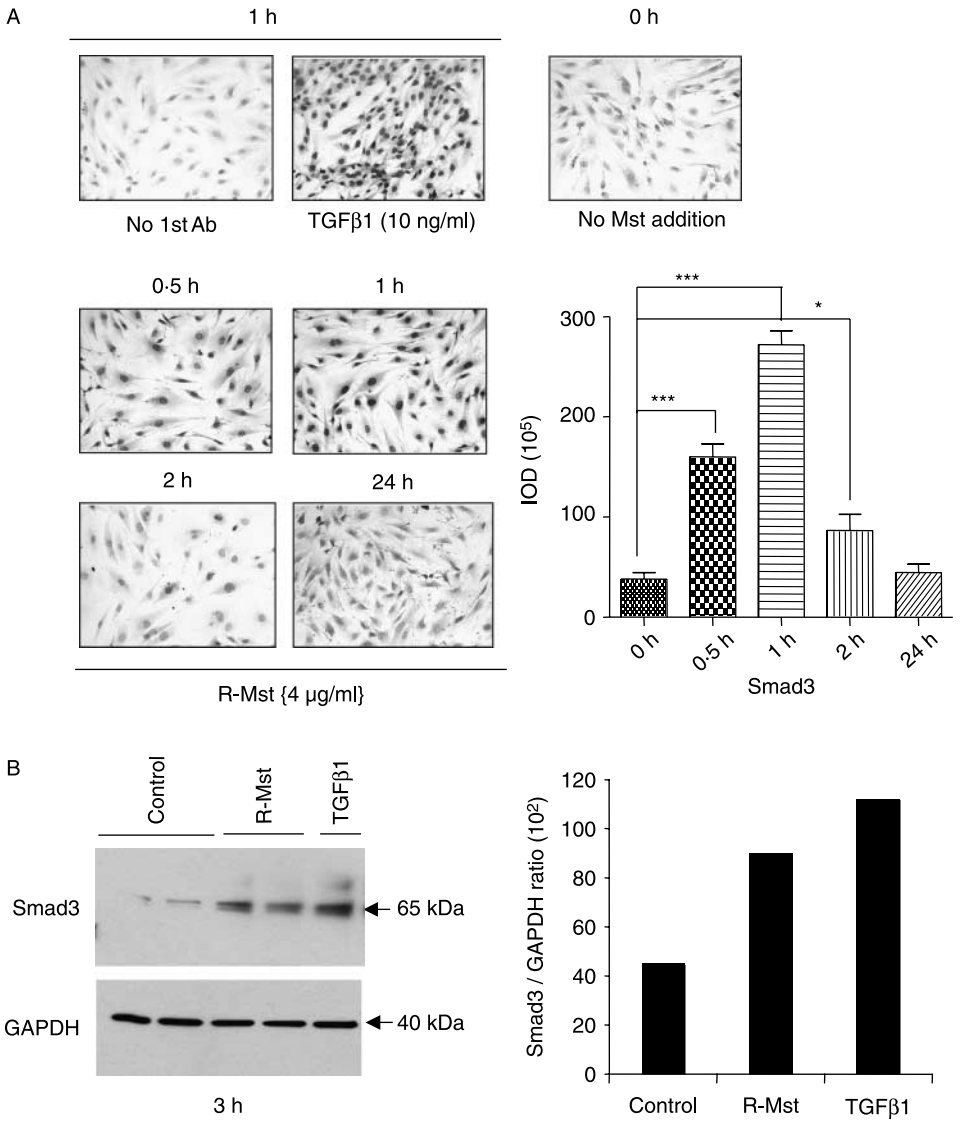


Figure 3 Myostatin stimulates the early expression of Smad3 protein in C3H10T1/2 cells. Azacytidine-treated cells were incubated on eight-well removable chambers in a time course manner and, for the 3 h incubation, on six-well plates, with or without myostatin, for the indicated periods. TGF-β1 was used as positive control. (A) Representative pictures of the immunodetection for Smad3 are presented (left) (200×), as well as the quantitative image analysis (right). (B) Western blot analysis was performed for the extracts from the 3-h incubation (left) and the corresponding densitometry analysis (right). Control; R-Mst, recombinant myostatin protein and TGF-β1 as positive control. Ab, antibody. Mean ± S.E.M. corresponds to experiments done in triplicate. *$P<0{\cdot}05$, ***$P<0{\cdot}001$. (A) 200×.

analysis of the band intensities (Fig. 7B, right). The transfection efficiency was estimated at about 60% by co-transfection of the myostatin construct with a reporter vector, pcDNA-EGFP (Fig. 7A). The two different plasmid constructs for the shRNA against myostatin mRNA decreased the expression of the myostatin band, as evidenced by the western blot analysis and the corresponding densitometry analysis (Fig. 7B). In parallel, there was considerable upregulation of PAI-1, visualized by the western blot analysis, and of the myostatin densitometry analysis, which was also blocked by the myostatin shRNA (Fig. 7C). A random RNA construct corresponding to the shRNA sequences for myostatin had previously been shown by our group to be inactive both *in vitro* (Artaza *et al.* 2005) and *in vivo* (Magee *et al.* 2006). This set of experiments confirmed that endogenous myostatin might act similarly to the exogenous myostatin protein.

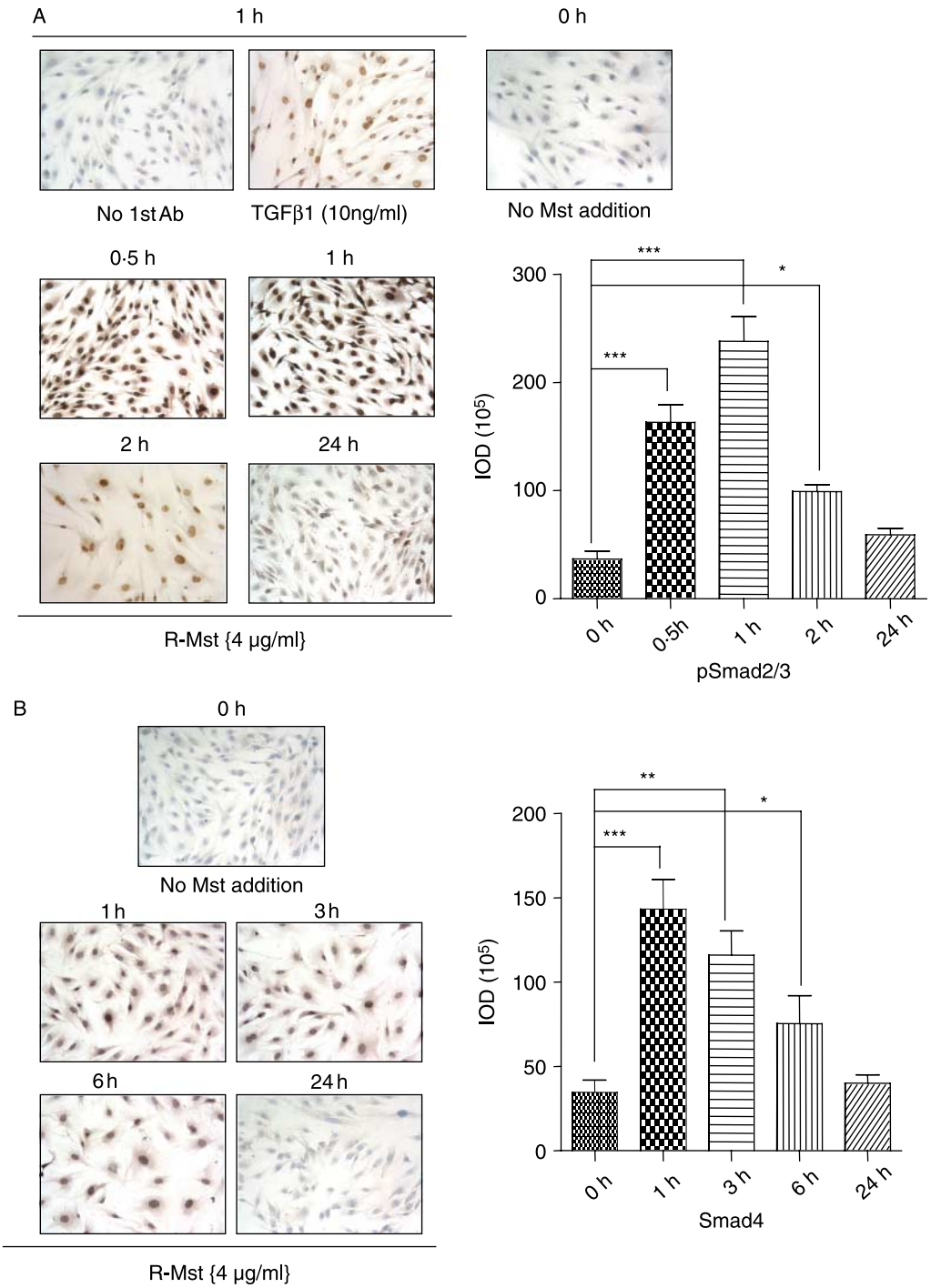


Figure 4 Time course of recombinant myostatin effects on the expression of the phosphorylated form of Smad2/3 protein (pSmad2/3) and Smad4 protein in C3H10T1/2 cells. Azacytidine-treated cells were incubated on eight-well removable chambers with or without myostatin, for the indicated periods. TGF-β1 was used as positive control. Representative pictures of the immunodetection are presented in left panels (200×), as well as the quantitative image analysis in the right panels. (A) pSmad2/3, (B) Smad4. Ab, antibody; R-Mst, recombinant myostatin protein. *$P<0{\cdot}05$, **$P<0{\cdot}01$, ***$P<0{\cdot}001$ (200×).

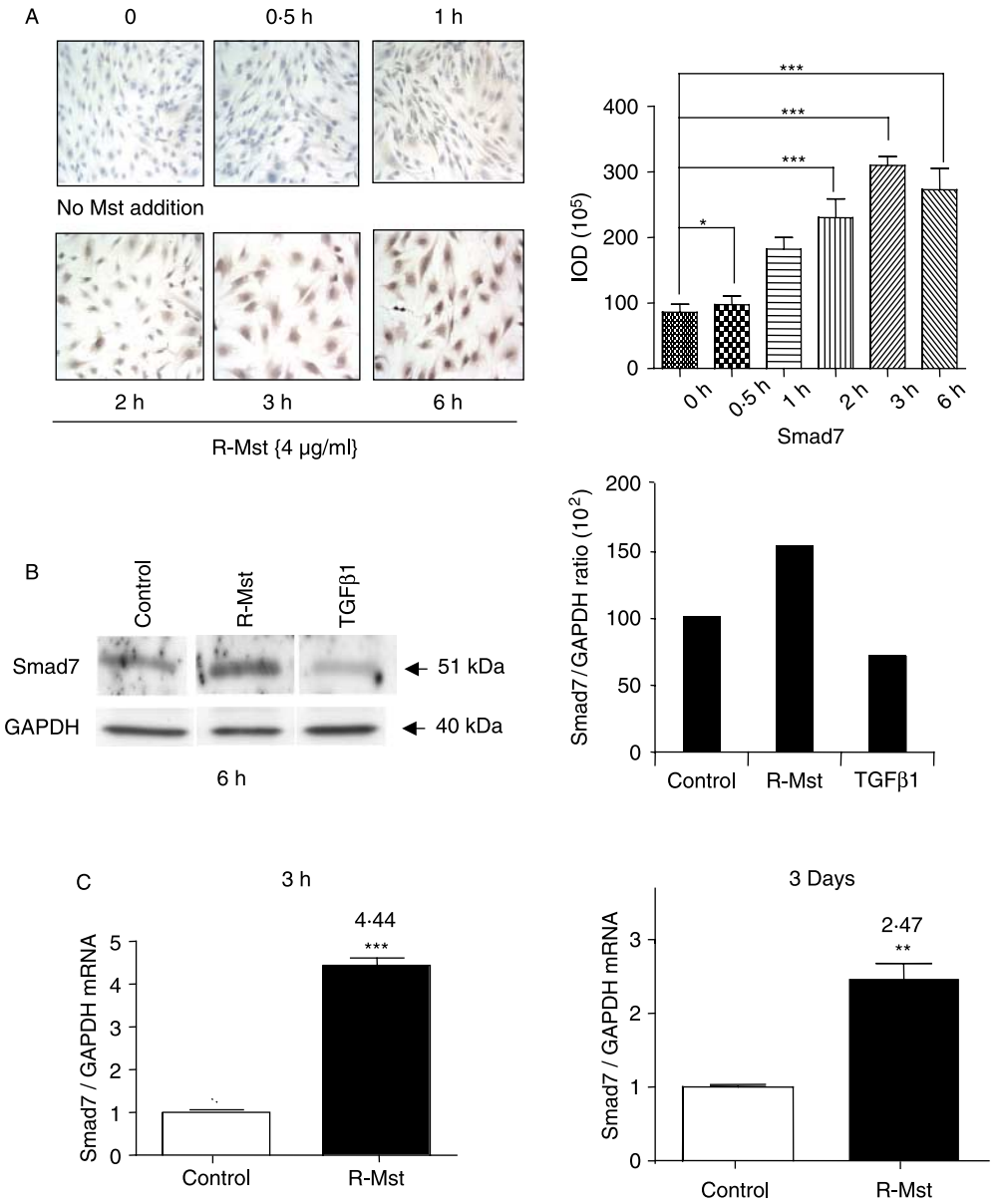


Figure 5 Time course of recombinant myostatin effects on the expression of the Smad7 gene in C3H10T1/2 cells. Azacytidine-treated cells were incubated on eight-well removable chambers in a time course manner, and, for the 3 h, 6 h, and 3 days incubations, on six-well plates, with or without myostatin. TGF-β1 was used as positive control. (A) Representative pictures of the immunodetection are presented (left), as well as the quantitative image analysis (right). (B) Western immunoblot analysis was performed for extracts from the 6-h incubation (left) and the corresponding densitometry analysis (right). (C) Total RNA isolation followed by real-time RT-PCR was applied in other aliquots for the 3-h and 3-day incubations normalized by GAPDH housekeeping gene. Ab, antibody; R-Mst, recombinant myostatin protein. Mean±S.E.M. corresponds to experiments done in triplicate. *$P<0{\cdot}05$, **$P<0{\cdot}01$, ***$P<0{\cdot}001$ (200×).

Discussion

The current study elucidates an important issue in the cell biology effects of myostatin, a protein that, in addition to its well-known role as a negative regulator of skeletal muscle mass and modulator of stem cell differentiation, acts as a profibrotic in this tissue (Engvall & Wewer 2003, McCroskery *et al.* 2005). This is consistent with the effects of other members of the TGF-β family such as activin and TGF-β1 in various tissues (Wada *et al.* 2004, Yamashita *et al.* 2004, Verrechia *et al.* 2006). Using the multipotent mesenchymal embryonic C3H10T1/2 cell line, it was possible in our

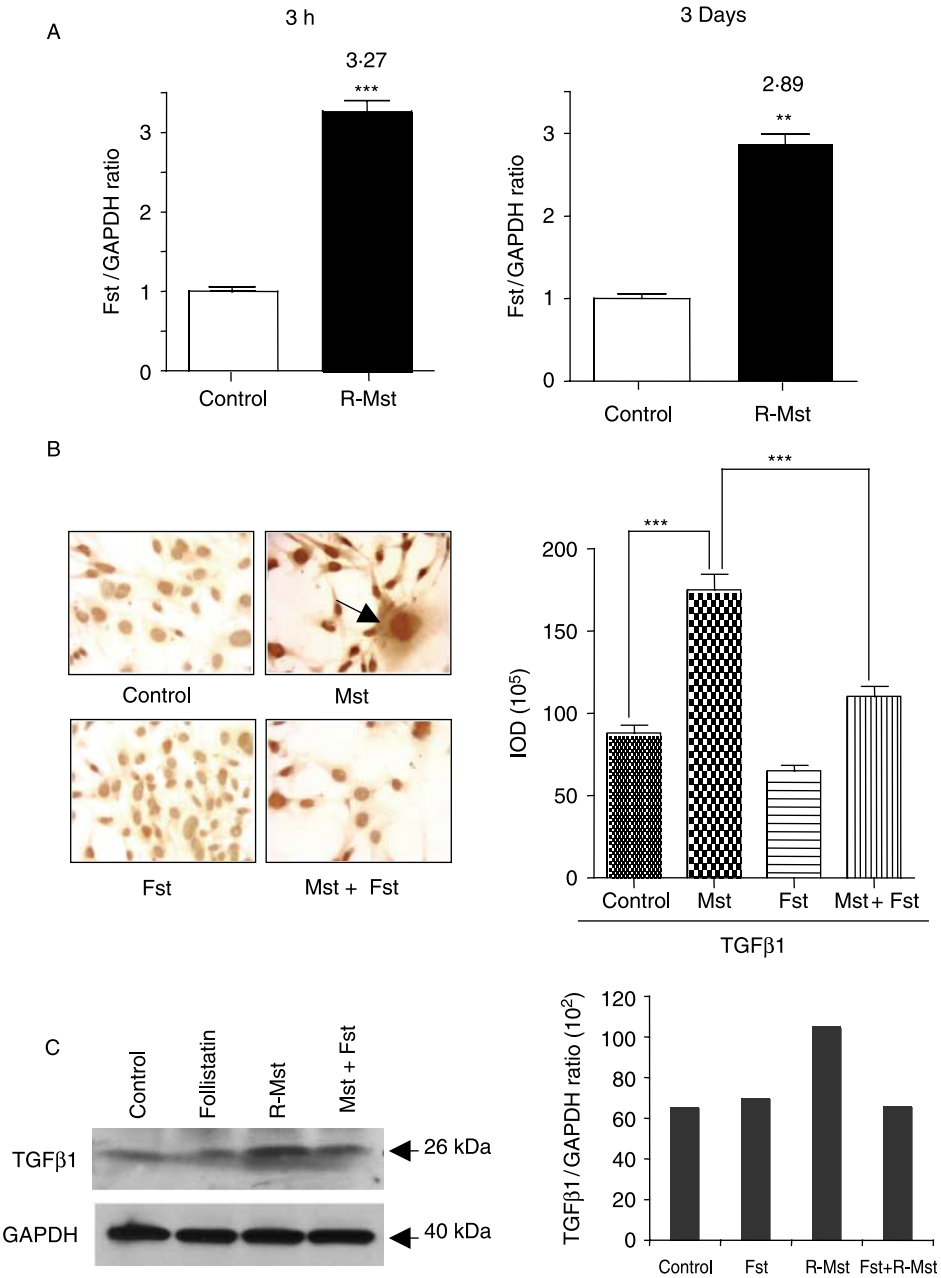


Figure 6 Effects of recombinant myostatin on follistatin and TGF-β1 expression, and modulation by follistatin of TGF-β1 protein expression. (A) Total RNA from the experiment of Fig. 5 was subjected to real-time RT-PCR for follistatin mRNA. (B) In a separate experiment, azacytidine-treated cells were incubated with either recombinant myostatin (4 μg/ml), TGF-β1 (5 ng/ml) or follistatin (0·5 μg/ml), for 4 days and subjected to immunocytochemistry and quantitative image analysis for TGF-β1 (200×). (C) Western immunoblot analysis for TGF-β1: R-Mst, recombinant myostatin protein; Fst, follistatin. $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$.

current work to demonstrate that myostatin did not affect paracrinely the differentiation of these cells into myofibroblasts (the typical fibrotic cells), and did not generate smooth muscle cells that are also potentially involved in extracellular matrix deposition when they switch to a fibrotic phenotype. However, myostatin was found to induce this switch in the overall multipotent and myofibroblast cell populations, as indicated by the stimulation in most cells within the culture of the expression TGF-β1 and another key profibrotic factor, PAI-1 (Eddy & Fogo 2006), and particularly by the ultimate

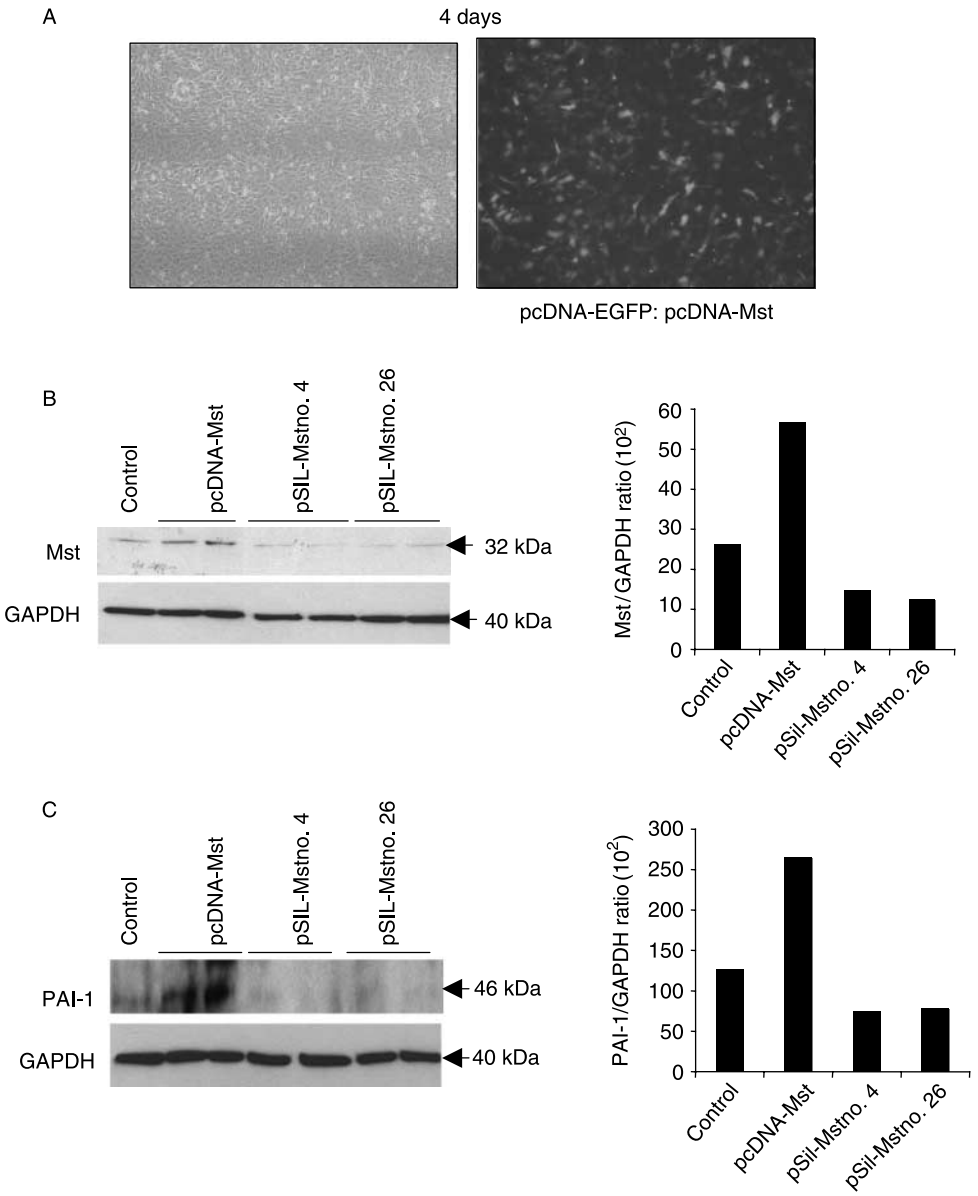


Figure 7 Effect of the modulation of the endogenous expression of myostatin on the expression of PAI-1. C3H10T1/2 cells were grown on six-well plates and transfected with either a reporter gene plasmid construct and a plasmid DNA construct encoding myostatin, or the latter construct or the one for myostatin shRNA, and analyzed after 4 days. (A) Fluorescent microscope, green filter (right), or regular light (left) of cells co-transfected with pcDNA-Mst and pcDNA-EGFP in order to check transfection efficiency (200×). (B) Western blot analysis of myostatin expression, with GAPDH as reference gene of cells transfected with: pcDNA-Mst-375; Mst siRNAs: pSiL-Mstno. 4 and pSiL Mstno. 26 (sequences no. 4 and no. 26) (left), densitometry evaluation of band intensities (right). (C) Western blot analysis of PAI-1 expression, as shown in (B).

products that define fibrosis – collagens I, III, and other isoforms (Bhogal *et al.* 2005, Attallah *et al.* 2007).

These processes were associated with the upregulation of the expression of Smad3 and 4, and the phosphorylation of Smad2 and 3, as expected from a member of the TGF-β family that signals through this pathway (Zhu *et al.* 2004, Kollias *et al.* 2006). An antifibrotic process was simultaneously elicited, as evidenced by the stimulation of the expression of a) the myostatin activity inhibitor, follistatin (Hill *et al.* 2002, Amthor *et al.* 2004, Kocamis *et al.* 2004), b) a Smad signaling inhibitor, Smad7 (Forbes *et al.* 2006), and c) a collagen breakdown inducer, matrix metalloproteinase 8 (MMP-8; Siller-Lopez *et al.* 2004). Follistatin did block the upregulation of TGF-β1 expression by myostatin, as shRNA against myostatin (Magee *et al.* 2006) inhibited the

expression of PAI-1, which in turn was stimulated autocrinely by the forced over-expression of myostatin. It is also well known that PAI-1 expression is upregulated by TGF-β1 (Otsuka *et al.* 2007).

An intriguing aspect of this study is that neither myostatin nor the well-known demethylating agent and differentiation inducer, azacytidine (Singh *et al.* 2003), affected the sizable number of C3H10T1/2 cells (7–8%) that expressed the typical myofibroblast marker ASMA, in the absence of any detectable expression of the smooth muscle cell marker, calponin. Myostatin acted differently from TGF-β1 in this respect, and in its inability to induce (at least in 10 days) the smooth muscle cell lineage, since TGF-β1 stimulates considerably the appearance of smooth muscle markers in this cell line at an earlier stage, acting through the Smad2/3 pathway (Sato *et al.* 2005, Chen *et al.* 2006).

The induction by exogenous recombinant myostatin protein of the expression of collagens I and III mRNA and protein is the hallmark of the acquisition of a fibrotic phenotype. This is likely to be mediated by the ActIIb receptor that binds myostatin and mediates its signaling, and which was detected in C3H10T1/2 cells (Artaza *et al.* 2007). The increase in the mRNA expression of other minor isoforms of collagen, particularly II and IV, and, to a lesser extent, collagen IX, by myostatin, agrees with what occurs in fibrotic conditions (Bhogal *et al.* 2005, Attallah *et al.* 2007). This may be due at least in part to the observed upregulation of TGF-β1 (Liu *et al.* 2006). This factor is known to upregulate myostatin expression (Budasz-Rwiderska *et al.* 2005). Reports are not available on the induction by myostatin of the other main vascular fibrotic factor, PAI-1, and we assume that the considerable upregulation observed may be either a direct effect of myostatin or is mediated by TGF-β1 (Otsuka *et al.* 2007).

In any case, the observed profibrotic effects of myostatin do not seem to be due to the appearance of a new specific cell lineage that would be very active in extracellular matrix deposition, and would differ from the rest of the cell population in this respect. Rather, profibrotic factor expression and collagen deposition may be mediated by the stimulation of the general multipotent fibroblast population to acquire this phenotype, with little conversion into myofibroblasts and none into smooth muscle cells. This would be similar to the differentiated smooth muscle cell transition from a 'contractile' to 'synthetic' phenotype (Budasz-Rwiderska *et al.* 2005). This transition has also been documented on myofibroblast cultures *in vivo* and *in vitro* (Hirose *et al.* 1999, Burstein *et al.* 2007, Darby & Hewitson 2007, Krieg *et al.* 2007), and myostatin should be considered, along with TGF-β1, as a factor eliciting this response.

We believe that the most significant findings of our study are the detection of an early 'antifibrotic' response simultaneous to the profibrotic phenotype induced by myostatin, as evidenced by the observed upregulation of Smad7, follistatin, and MMP8. The upregulation of Smad7 by myostatin was already observed in an elegant study where myostatin induced the expression of Smad7 in C2C12 myoblasts. The latter in turn inhibited myostatin promoter activity, thus suggesting that myostatin autoregulates its expression by feedback loop through Smad7 (Forbes *et al.* 2006), which involves the interaction of Smad2/3 with the Smad7 promoter (Zhu *et al.* 2004). The fact that Smad7 abrogates myostatin – but not TGF-β1-mediated repression of myogenesis (Kollias *et al.* 2007) – raises the question of why myofibroblast differentiation was not triggered by the myostatin-induced TGF-β1 expression in our experiments. It seems that either this expression was too low to trigger C3H10T1/2 cell differentiation, or the Smad7 induction by myostatin was blocked in these cells through counteracting the Smad2/3 upregulation. Myostatin does inhibit myoblast progression into myotubes in C2C12 myoblasts via Smad3 phosphorylation (Langley *et al.* 2002).

The upregulation of follistatin expression by myostatin does not seem to have been reported before, although TGF-β1 through the Smad protein potentiates the stimulatory effects of tumor necrosis factor-α (TNF-α) on the activity of a follistatin-related gene promoter (Bartholin *et al.* 2007), and TNF-α is associated with fibrotic processes (Yoshimura 2006). TGF-β1 also directly upregulates follistatin expression in bovine granulosa cells (Fazzini *et al.* 2006). As stated previously, follistatin in turn inactivates myostatin, and is a well-known antifibrotic agent (Sulyok *et al.* 2004, Wada *et al.* 2004). Our findings are consistent in terms of indicating that treatment with follistatin counteracted the upregulation of TGF-β1 expression exerted by recombinant myostatin in our culture.

Smad7 and follistatin inhibit myostatin signaling and activity respectively, and therefore act as a feedback mechanism against myostatin profibrotic effects, demonstrated by the fact that the C3H10T1/2 cells reacted to myostatin by upregulating MMP-8. This metalloproteinase, like MMP-1 or MMP-13, cleaves collagen at a single site and renders it susceptible to degradation by other MMPs and proteases. An adenoviral construct for a cDNA encoding MMP-8 over-expressed the MMP-8 pro-collagenase in rat models of liver fibrosis, which was then endogenously activated and led to a reversion of the process (Siller-Lopez *et al.* 2004). The MMP-8 upregulation exerted by myostatin in our system therefore appears to be an attempt to degrade collagens I and III deposited as a result of incubation with myostatin. This is sort of a second stage of defense to complement the feedback mechanism that inactivates myostatin.

The interplay of profibrotic and antifibrotic processes observed in the current study with myostatin suggests that counteracting myostatin might be a potentially effective therapy against fibrosis, in addition to that based on the use of decorin (Li *et al.* 2004), Smad7 cDNA (Forbes *et al.* 2006), and follistatin (Aoki *et al.* 2005, Patella *et al.* 2006), against TGF-β1/activin A/Smad signaling, or the use of agents such as deacetylase inhibitors that induce follistatin (Iezzi *et al.* 2004). This approach of targeting myostatin may aim to downregulate myostatin expression, like the shRNA against

myostatin applied in the current *in vitro* study, and that we previously used *in vitro* and *in vivo* to promote myogenesis (Magee *et al*. 2006). Further experimental work is needed on the potential application of these inhibitors to discriminate the relative contribution of myostatin, TGF-β1, and activin to fibrotic processes. It is also important to elucidate whether the Smad pathway is the single downstream signaling for these effectors in the acquisition of the fibrotic phenotype by terminally differentiated cells, and if so, whether Smad7 indeed acts specifically on myostatin in this respect. In addition, despite our results with the C3H10T1/2 cells, it is uncertain whether myostatin *in vivo* triggers the differentiation of endogenous or circulating stem cells to myofibroblasts, as does TGF-β1. If this would be the case, then the anti-myostatin strategy may also block an additional profibrotic mechanism operating through stem cell lineage commitment. However, the fact that myostatin stimulates the switch of myofibroblast to a fibrotic phenotype suggests that this by itself may be the main cellular target for its profibrotic effects, since myofibroblasts play such a fundamental role in fibrosis and scarring (Darby & Hewitson 2007).

Acknowledgements

This work was supported by NIH/MBRS Score Program 3S06-GM068510-02S21 (J A & J T), NIH/NCRR 5U54 RR019234-05 (J A), NIH/National Center on Minority and Health Disparities 1P20 MD000545-02 (J A & M G F), Department of Defense PR064756 (N G C), and NIH/RCMI G12R R003026. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

Amthor H, Nicholas G, McKinnell I, Kemp CF, Sharma M, Kambadur R & Patel K 2004 Follistatin complexes myostatin and antagonises myostatin-mediated inhibition of myogenesis. *Developmental Biology* **270** 19–30.

Aoki F, Kurabayashi M, Hasegawa Y & Kojima I 2005 Attenuation of bleomycin-induced pulmonary fibrosis by follistatin. *American Journal of Respiratory and Critical Care Medicine* **172** 713–720.

Artaza JN, Bhasin S, Mallidis C, Taylor W, Ma K & Gonzalez-Cadavid NF 2002 Endogenous expression and localization of myostatin and its relation to myosin heavy chain distribution in C2C12 skeletal muscle cells. *Journal of Cellular Physiology* **190** 170–179.

Artaza JN, Bhasin S, Magee TR, Reisz-Porszasz S, Shen R, Groome NP, Meerasahib MF & Gonzalez-Cadavid NF 2005 Myostatin inhibits myogenesis and promotes adipogenesis in C3H 10T(1/2) mesenchymal multipotent cells. *Endocrinology* **146** 3547–3557.

Artaza JN, Reisz-Porszasz S, Dow JS, Kloner RA, Tsao J, Bhasin S & Gonzalez-Cadavid NF 2007 Alterations in myostatin expression are associated with changes in cardiac left ventricular mass but not ejection fraction in the mouse. *Journal of Endocrinology* **194** 63–76.

Ask K, Martin GE, Kolb M & Gauldie J 2006 Targeting genes for treatment in idiopathic pulmonary fibrosis: challenges and opportunities, promises and pitfalls. *Proceedings of the American Thoracic Society* **3** 389–393 (Review).

Atkinson BL, Fantle KS, Benedict JJ, Huffer WE & Gutierrez-Hartmann A 1997 Combination of osteoinductive bone proteins differentiates mesenchymal C3H/10T1/2 cells specifically to the cartilage lineage. *Journal of Cellular Biochemistry* **65** 325–339.

Attallah AM, Mosa TE, Omran MM, Abo-Zeid MM, El-Dosoky I & Shaker YM 2007 Immunodetection of collagen types I, II, III, and IV for differentiation of liver fibrosis stages in patients with chronic HCV. *Journal of Immunoassay and Immunochemistry* **28** 155–168.

Bartholin L, Guindon S, Martel S, Corbo L & Rimokh R 2007 Identification of NF-κB responsive elements in follistatin related gene (FLRG) promoter. *Gene* **393** 153–162.

Bhogal RK, Stoica CM, McGaha TL & Bona CA 2005 Molecular aspects of regulation of collagen gene expression in fibrosis. *Journal of Clinical Immunology* **25** 592–603.

Budasz-Rwiderska M, Jank M & Motyl T 2005 Transforming growth factor-beta1 upregulates myostatin expression in mouse C2C12 myoblasts. *Journal of Physiology and Pharmacology* **56** (Suppl 3) 195–214.

Burstein B, Qi XY, Yeh YH, Calderone A & Nattel S 2007 Atrial cardiomyocyte tachycardia alters cardiac fibroblast function: a novel consideration in atrial remodeling. *Cardiovascular Research* **76** 442–452.

Chen S, Crawford M, Day RM, Briones VR, Leader JE, Jose PA & Lechleider RJ 2006 RhoA modulates Smad signaling during transforming growth factor-beta induced smooth muscle differentiation. *Journal of Biological Chemistry* **281** 1765–1770.

Darby IA & Hewitson TD 2007 Fibroblast differentiation in wound healing and fibrosis. *International Review of Cytology* **257** 143–179.

Eddy AA & Fogo AB 2006 Plasminogen activator inhibitor-1 in chronic kidney disease: evidence and mechanisms of action. *Journal of the American Society of Nephrology* **17** 2999–3012.

Engvall E & Wewer UM 2003 The new frontier in muscular dystrophy research: booster genes. *FASEB Journal* **17** 1579–1584.

Fazzini M, Vallejo G, Colman-Lerner A, Trigo R, Campo S, Baranao JL & Saragueta PE 2006 Transforming growth factor beta1 regulates follistatin mRNA expression during *in vitro* bovine granulosa cell differentiation. *Journal of Celluar Physiology* **207** 40–48.

Feldman BJ, Streeper RS, Farese RV, Jr & Yamamoto KR 2006 Myostatin modulates adipogenesis to generate adipocytes with favorable metabolic effects. *PNAS* **103** 15675–15680.

Fischer L, Boland G & Tuan RS 2002 Wnt-3A enhances bone morphogenetic protein-2-mediated chondrogenesis of murine C3H10T1/2 mesenchymal cells. *Journal of Biological Chemistry* **277** 30870–30878.

Forbes D, Jackman M, Bishop A, Thomas M, Kambadur R & Sharma M 2006 Myostatin auto-regulates its expression by feedback loop through Smad7 dependent mechanism. *Journal of Celluar Physiology* **206** 264–272.

Gharaee-Kermani M, Gyetko MR, Hu B & Phan SH 2007 New insights into the pathogenesis and treatment of idiopathic pulmonary fibrosis: a potential role for stem cells in the lung parenchyma and implications for therapy. *Pharmacological Research* **24** 819–841.

Hamrick MW, Shi X, Zhang W, Pennington C, Thakore H, Haque M, Kang B, Isales CM, Fulzele S & Wenger KH 2007 Loss of myostatin (GDF8) function increases osteogenic differentiation of bone marrow-derived mesenchymal stem cells but the osteogenic effect is ablated with unloading. *Bone* **40** 1544–1553.

Henderson NC & Iredale JP 2007 Liver fibrosis: cellular mechanisms of progression and resolution. *Clinical Science* **112** 265–280.

Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM & Qiu Y 2002 The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. *Journal of Biological Chemistry* **277** 40735–40741.

Hirose M, Kosugi H, Nakazato K & Hayashi T 1999 Restoration to a quiescent and contractile phenotype from a proliferative phenotype of myofibroblast-like human aortic smooth muscle cells by culture on type IV collagen gels. *Journal of Biochemistry* **125** 991–1000.

Iezzi S, Di Padova M, Serra C, Caretti G, Simone C, Maklan E, Minetti G, Zhao P, Hoffman EP, Puri PL *et al*. 2004 Deacetylase inhibitors increase muscle cell size by promoting myoblast recruitment and fusion through induction of follistatin. *Developmental Cell* **6** 673–684.

Iredale JP 2007 Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *Journal of Clinical Investigation* **117** 539–548.

Jasuja R, Ramaraj P, Mac RP, Singh AB, Storer TW, Artaza J, Miller A, Singh R, Taylor WE, Lee ML *et al.* 2005 Delta-4-androstene-3, 17-dione binds androgen receptor, promotes myogenesis *in vitro*, and increases serum testosterone levels, fat-free mass, and muscle strength in hypogonadal men. *Journal of Clinical Endocrinology and Metabolism* **90** 855–863.

Kisseleva T & Brenner DA 2006 Hepatic stellate cells and the reversal of fibrosis. *Journal of Gastroenterology and Hepatology* **21** (Suppl 3) S84–S87.

Kocamis H, Gulmez N, Aslan S & Nazli M 2004 Follistatin alters myostatin gene expression in C2C12 muscle cells. *Acta Veterinaria Hungarica* **52** 135–141.

Kollias HD, Perry RL, Miyake T, Aziz A & McDermott JC 2006 Smad7 promotes and enhances skeletal muscle differentiation. *Molecular and Cellular Biology* **26** 6248–6260.

Krieg T, Abraham D & Lafyatis R 2007 Fibrosis in connective tissue disease: the role of the myofibroblast and fibroblast–epithelial cell interactions. *Arthritis Research and Therapy* **9** (Suppl 2) S4.

Langley B, Thomas M, Bishop A, Sharma M, Gilmour S & Kambadur R 2002 Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. *Journal of Biological Chemistry* **277** 49831–49840.

Lee SJ 2004 Regulation of muscle mass by myostatin. *Annual Review of Cell and Developmental Biology* **20** 61–86.

Lee SJ & McPherron AC 2001 Regulation of myostatin activity and muscle growth. *PNAS* **98** 9306–9311.

Li Y, Foster W, Deasy BM, Chan Y, Prisk V, Tang Y, Cummins J & Huard J 2004 Transforming growth factor-beta1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: a key event in muscle fibrogenesis. *American Journal of Pathology* **164** 1007–1019.

Liu Y 2006 Renal fibrosis: new insights into the pathogenesis and therapeutics. *Kidney International* **69** 213–217.

Liu X, Hu H & Yin JQ 2006 Therapeutic strategies against TGF-β signaling pathway in hepatic fibrosis. *Liver International* **26** 8–22.

Magee TR, Artaza JN, Ferrini MG, Zuniga FI, Cantini L, Reisz-Porszasz S, Rajfer J & Gonzalez-Cadavid NF 2006 Myostatin short interfering hairpin RNA gene transfer increases skeletal muscle mass. *Journal of Gene Medicine* **8** 1171–1181.

Mauviel A 2005 Transforming growth factor-beta: a key mediator of fibrosis. *Methods in Molecular Medicine* **117** 69–80.

McCroskery S, Thomas M, Platt L, Hennebry A, Nishimura T, McLeay L, Sharma M & Kambadur R 2005 Improved muscle healing through enhanced regeneration and reduced fibrosis in myostatin-null mice. *Journal of Cell Science* **118** 3531–3541.

Otsuka G, Stempien-Otero A, Frutkin AD & Dichek DA 2007 Mechanisms of TGF-beta1-induced intimal growth: plasminogen-independent activities of plasminogen activator inhibitor-1 and heterogeneous origin of intimal cells. *Circulation Research* **100** 1300–1307.

Patella S, Phillips DJ, Tchongue J, de Kretser DM & Sievert W 2006 Follistatin attenuates early liver fibrosis: effects on hepatic stellate cell activation and hepatocyte apoptosis. *American Journal of Physiology. Gastrointestinal and Liver Physiology* **290** G137–G144.

Qi W, Chen X, Poronnik P & Pollock CA 2006 The renal cortical fibroblast in renal tubulo interstitial fibrosis. *International Journal of Biochemistry and Cell Biology* **38** 1–5.

Ruiz-Ortega M, Rodriguez-Vita J, Sanchez-Lopez E, Carvajal G & Egido J 2007 TGF-beta signaling in vascular fibrosis. *Cardiovascular Research* **74** 196–206.

Sato M, Kawai-Kowase K, Sato H, Oyama Y, Kanai H, Ohyama Y, Suga T, Maeno T, Aoki Y, Tamura J *et al.* 2005 c-Src and hydrogen peroxide mediate transforming growth factor-beta1-induced smooth muscle cell-gene expression in 10T1/2 cells. *Arteriosclerosis, Thrombosis, and Vascular Biology* **25** 341–347.

Schmittwolf C, Kirchhof N, Jauch A, Dürr M, Harder F, Zenke M & Müller AM 2005 *In vivo* haematopoietic activity is induced in neurosphere cells by chromatin-modifying agents. *EMBO Journal* **24** 554–566.

Siller-Lopez F, Sandoval A, Salgado S, Salazar A, Bueno M, Garcia J, Vera J, Galvez J, Hernandez I, Ramos M *et al.* 2004 Treatment with human metalloproteinase-8 gene delivery ameliorates experimental rat liver cirrhosis. *Gastroenterology* **126** 1122–1133.

Singh R, Artaza JN, Taylor WE, Gonzalez-Cadavid NF & Bhasin S 2003 Androgens stimulate myogenic differentiation and inhibit adipogenesis in C3H 10T1/2 pluripotent cells through an androgen receptor-mediated pathway. *Endocrinology* **144** 5081–5088.

Singh R, Artaza JN, Taylor WE, Braga M, Yuan X, Gonzalez-Cadavid NF & Bhasin S 2006 Testosterone inhibits adipogenic differentiation in 3T3-L1 cells: nuclear translocation of androgen receptor complex with beta-catenin and T-cell factor 4 may bypass canonical Wnt signaling to down-regulate adipogenic transcription factors. *Endocrinology* **147** 141–154.

Sulyok S, Wankell M, Alzheimer C & Werner S 2004 Activin: an important regulator of wound repair, fibrosis, and neuroprotection. *Molecular and Cellular Endocrinology* **225** 127–132.

Taylor SM & Jones PA 1979 Multiple new phenotypes induced in 10T1/2 and 2T3 cells treated with azacytidine. *Cell* **17** 769–779.

Taylor WE, Bhasin S, Artaza J, Byhower F, Azam M, Willard DH Jr, Kull FC Jr & Gonzales-Cadavid N 2001 Myostatin inhibits cell proliferation and protein synthesis in C2C12 muscle cells. *American Journal of Physiology, Endocrinology and Metabolism* **280** E221–E228.

Tsuchida K 2004 Activins, myostatin and related TGF-beta family members as novel therapeutic targets for endocrine, metabolic and immune disorders. *Current Drug Targets. Immune, Endocrine and Metabolic Disorders* **4** 157–166.

Verrecchia F, Mauviel A & Farge D 2006 Transforming growth factor β signaling through the Smad proteins: role in systemic sclerosis. *Autoimmune Reviews* **5** 563–569.

Wada W, Kuwano H, Hasegawa Y & Kojima I 2004 The dependence of transforming growth factor-beta-induced collagen production on autocrine factor activin A in hepatic stellate cells. *Endocrinology* **145** 2753–2759.

Wang W, Koka V & Lan HY 2005 Transforming growth factor-beta and Smad signalling in kidney diseases. *Nephrology* **10** 48–56.

Willis BC, duBois RM & Borok Z 2006 Epithelial origin of myofibroblasts during fibrosis in the lung. *Proceedings of the American Thoracic Society* **3** 377–382.

Wilson EM & Rotwein P 2006 Control of MyoD function during initiation of muscle differentiation by an autocrine signaling pathway activated by insulin-like growth factor-II. *Journal of Biological Chemistry* **281** 62–71.

Wynn TA 2007 Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *Journal of Clinical Investigation* **117** 524–529.

Yamashita S, Maeshima A, Kojima I & Nojima Y 2004 Activin A is a potent activator of renal interstitial fibroblasts. *Journal of the American Society of Nephrology* **15** 91–101.

Yoshimura A 2006 Signal transduction of inflammatory cytokines and tumor development. *Cancer Science* **97** 439–447.

Zhu X, Topouzis S, Liang LF & Stotish RL 2004 Myostatin signaling through Smad2, Smad3 and Smad4 is regulated by the inhibitory Smad7 by a negative feedback mechanism. *Cytokine* **26** 262–272.

Received in final form 4 October 2007
Accepted 7 November 2007
Made available online as an Accepted Preprint
7 November 2007

Profibrotic Role of Myostatin in Peyronie's Disease

Liliana P. Cantini, ••,*¶ Monica G. Ferrini, ••,*‡§ Dolores Vernet, ••,* Thomas R. Magee, ••,*‡
Ansha Qian, ••,* Robert A. Gelfand, ••,* Jacob Rajfer, ••,*†‡ and Nestor F. Gonzalez-Cadavid, PhD*†‡§

*Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Urology Research Laboratory, Los Angeles, CA, USA; †Division of Urology, Harbor-UCLA Medical Center, Torrance, CA, USA; ‡Department of Urology, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA; §Department of Internal Medicine, The Charles Drew University, Los Angeles, CA, USA; ¶Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela

DOI: 10.1111/j.1743-6109.2008.00847.x

ABSTRACT

Introduction. The primary histologic finding in many urologic disorders, including Peyronie's disease (PD), is fibrosis, mainly mediated by the transforming growth factor β1 (TGFβ1).

Aim. To determine whether another member of the TGFβ family, myostatin, (i) is expressed in the human PD plaque and normal tunica albuginea (TA), their cell cultures, and the TGFβ1-induced PD lesion in the rat model; (ii) is responsible for myofibroblast generation, collagen deposition, and plaque formation; and (iii) mediates the profibrotic effects of TGFβ1 in PD.

Methods. Human TA and PD tissue sections, and cell cultures from both tissues incubated with myostatin and TGFβ1 were subjected to immunocytochemistry for myostatin and α-smooth muscle actin (ASMA). The cells were assayed by western blot, RT-PCR, and ribonuclease protection. Myostatin cDNA and shRNA were injected, with or without TGFβ1, in the rat penile TA, and plaque size was estimated by Masson.

Main Outcome Measures. Myostatin expression in the human TA, the PD plaque, and their cell cultures, and myostatin effects on the PD-like plaque in the rat.

Results. A threefold overexpression of myostatin was found in the PD plaque as compared with the TA. In PD cells, myostatin expression was mainly in the myofibroblasts, and in the TA cells, it increased upon passage paralleling myofibroblast differentiation and was up-regulated by TGFβ1. Myostatin or its cDNA construct increased the myofibroblast number and collagen in TA cells. Myostatin was detected in the TGFβ1-induced PD-like plaque of the rat partly in the myofibroblasts, and in the TA. Myostatin cDNA injected in the TA induced a plaque and intensified the TGFβ1 lesion, which was not reduced by myostatin shRNA.

Conclusions. Myostatin is overexpressed in the PD plaque, partly because of myofibroblast generation. Although myostatin induces a plaque in the rat TA, it does not appear to mediate the one triggered by TGFβ1, thus suggesting that both proteins act concurrently and that therapy should target their common downstream effectors. **Cantini LP, Ferrini MG, Vernet D, Magee TR, Qian A, Gelfand RA, Rajfer J, and Gonzalez-Cadavid NF. Profibrotic role of myostatin in Peyronie's disease. J Sex Med **;**:**–**.**

Key Words. Fibrosis; Fibroblasts; Myofibroblasts; TGFβ1; Stem Cells; Penis

Introduction

Fibrosis refers to an excessive deposition of both collagen fibers and extracellular matrix combined with a relative decrease of cell number. This process is now considered to be at the root of many urologic disorders such as erectile dysfunction, specifically corporal veno-occlusive dysfunction [1–6], overactive bladder, and benign prostatic hyperplasia [7–9], as well as certain nephropathies [10,11]. One of the best recognized forms of fibrosis is Peyronie's disease (PD), which is characterized by a distinct circumscribed lesion in the penile tunica albuginea (TA) [12,13]. This condition is rather prevalent, impairs the quality of life of both patients and their partners, and is essentially refractory to any medical treatment. It has been hypothesized that the PD plaque results from

microtrauma during the sexual act, may cause severe penile curvature and pain upon erection, and is often associated with erectile dysfunction.

One of the fibrotic factors that seems to be involved in PD, as well as other localized and diffuse fibroses, is the transforming growth factor β1 (TGFβ1) [14,15], a cytokine that elicits collagen deposition and the generation of myofibroblasts, the main cell type involved in scarring and fibrosis [16,17]. The expression of TGFβ1 is enhanced in the PD plaque as compared with the normal TA. A widely employed animal model of this condition is based on the injection of this agent into the TA of the rat, leading to the development, after 6–7 weeks, of a lesion that histologically resembles the human plaque in terms of collagen deposition, PAI-1 expression, oxidative stress, and other fibrotic markers [18–23]. Tunical TGFβ1 is up-regulated in another rat model of PD, where the TA injury is induced by the injection of fibrin into the TA [23–25]. This model reproduces the fibrinogen transvasation that occurs with microtrauma to the TA in human. Conversely, with interventions that reduce the tunical fibrosis in this fibrin-induced model of PD, TGFβ1 is down-regulated [25].

However, TGFβ1 is not the only member of the large TGFβ super family of growth and differentiation factors (GDFs) that have been implicated as fibrotic agents, as activin, inhibin, and some bone morphogenic proteins (BMPs) are known to induce fibrosis, all converging through downstream signaling by the Smad pathway and presumably, also via the connective tissue growth factor (CTGF) [16,26–29]. More recently, another family member, myostatin, also known as the GDF-8, the only well-identified negative regulator of skeletal muscle mass [30–32], has been proposed not only as an inhibitor of myofiber formation but also as an inducer of fibrosis in skeletal muscle [33–36]. Specifically, (i) myostatin stimulates fibroblast proliferation in vitro and induces differentiation of fibroblasts into myofibroblasts; (ii) TGFβ1 and myostatin stimulate each other's expression or secretion in vivo; (iii) myostatin knockout mice develop significantly less fibrosis when compared with wild-type mice following muscle injury; and (iv) both members of the TGFβ family colocalize in myofibers in the early stages of muscle injury [36].

In turn, myostatin intensifies the fibrotic phenotype of myofibroblasts differentiated from a multipotent mouse cell line of embryonic origin, the C3H 10T1/2, but not the preceding stage in its differentiation pathway into myofibroblasts [37]. This contrasts with the fact that myostatin does modulate myogenic and adipogenic differentiation of the parental cell line [38]. Several tools allow to investigate more mechanistically the role of myostatin in fibrosis vis-à-vis TGFβ1: cDNAs encoding the full-length 375 amino acid myostatin protein and its processed 110 amino acid carboxy terminus, as well as the respective recombinant proteins, and an shRNA that breaks down myostatin mRNA and elicits in vivo a functional response by increasing muscle mass [31,37–40].

In the current work, we have studied whether myostatin is expressed in the normal human penile TA, the human PD fibrotic plaque, in their respective cell cultures, and in the corresponding tissues from the rat model for this condition. We have also examined whether myostatin is expressed in human TA and PD myofibroblasts and whether it can induce their generation in vitro, stimulate collagen synthesis, and enhance in vivo the PD-like plaque triggered in the rat TA by TGFβ1, and whether myostatin plays any role in the aforementioned effects of TGFβ1.

Materials and Methods

Human Tissues and Cell Cultures

Tissue sections, and tissue homogenates preserved at –80°C, derived from one of our previous studies [19] were used. Normal TA was obtained from non-PD patients who were undergoing a penectomy because of either penile cancer or penile prosthesis surgery (N = 4), and the PD plaque was harvested from patients with PD (N = 8) who underwent a surgical procedure to remove the plaque. All procedures were IRB-approved, and a written informed consent was obtained. Fragments of the tissue had been stored for 24 hours in "RNAlater" (Ambion, Inc., Austin, TX, USA), for RNA analysis, in 4% formalin for histochemistry and immunohistochemistry, or in a culture medium (Dulbecco's modified essential medium [DMEM]/10% fetal calf serum) for protein analysis. Other portions were frozen at –80°C until further use, whereas the fixed tissues were stored at 4°C in PBS until paraffin embedding.

Fibroblast primary cultures containing stem cells were originally obtained from another study [41,42] and maintained in our laboratory either under liquid nitrogen or under cell culture passage. These cells were derived from fragments of human TA from non-PD patients (N = 3) undergoing penile prosthesis surgery or from

plaque tissue isolated from PD patients (N = 4) as above. A written informed consent was also obtained under IRB approval. Briefly, each specimen was washed in Hanks solution, minced in fibroblast growth medium-2 (FGM; Cambrex Inc., Walkersville, MD, USA) containing 20% fetal bovine serum (FBS), and plated onto a 25-cm^2 culture flask. Fragments were left undisturbed until attachment for about 1 week, and once the monolayer was starting to develop, they were removed. Medium with 10% serum was replaced once a week, and when cells achieved approximately 80% confluence (3–4 weeks), they were trypsinized and split onto three 10-cm plates. The cells were allowed to grow again to 80% confluence, with the medium changed twice weekly. The cells collected from this passage were considered as passage 1. Successive passages were performed at 1:3 split ratio, and the cells were used at passages 1–15. The purity of these cultures was established by immunocytochemistry for the fibroblast marker vimentin, which showed 100% staining as previously described, with virtually no TA cells positive for α-smooth muscle actin (ASMA) [20,41].

For the experiments, after trypsination and centrifugation the cell pellet from one plate was suspended and plated at 25–35% confluence on 8-well removable chamber plates (for immunocytochemistry), 12-well plates (for protein homogenates), or 6-well plates (for RNA isolation) and allowed to grow in either FGM or osteogenic medium (OM) supplemented with 10% FBS [43] for the indicated periods (usually 2 or 4 weeks). OM consisted of DMEM with 0.1 μM dexamethasone, 50 μM ascorbate-2 phosphate, and 10 mM β-glycerophosphate. In certain experiments, TGFβ1 was added at the indicated concentrations. All experiments were in duplicate or triplicate.

Myostatin Protein and Adenoviral cDNA Construct

Two human myostatin recombinant proteins, with identical amino acid sequence to the mouse counterparts, were used for cell incubations. They correspond to the 375 amino acid full sequence (Mst375) and to the 110 amino acid carboxy-terminus cleavage product claimed to be the final processed myostatin protein (Mst110). Each was prepared as described [31,39].

The construction of adenovirus expressing the mouse myostatin full-length cDNA under the CMV promoter (AdV-CMV-Mst375) was carried out as follows: the mouse myostatin cDNA was initially cloned into the donor plasmid pDNR-CMV using the Adeno-X Expression System II Kit (Clontech, Palo Alto, CA, USA). The myostatin sequence was generated by PCR from a previously cloned mouse myostatin plasmid (pcDNA3.1-myostatin) [40], by using primers located at the 5′ and 3′ regions. The 5′ ends of each primer have homology to the pDNR-CMV vector in order to facilitate recombinational cloning into the vector. PCR amplification was done in a reaction mix consisting of 1× HD Advantage polymerase buffer, 0.2 mM dNTP mix, 200 pmol of each primer, 50 ng of myostatin plasmid template, and 1 unit of Advantage HD polymerase (Clontech) in a total volume of 25 μL. The reaction consisted of 30 cycles of 94°C for 15 seconds, 60°C for 15 seconds, 72°C for 1 minute, followed by a final 72°C incubation for 10 minutes. PCR fragment was agarose gel purified and then recombined into the pDNR-CMV donor plasmid using the BD In-Fusion cloning kit as per manufacturer's instructions (Clontech). Recombinant plasmids were transformed into DH5-alpha competent *Escherichia coli* and clones verified by DNA restriction enzyme analysis and DNA sequencing resulting in plasmid pDNR-CMV-Mst375.

Then, the Mst375 cDNA was recombined into pLP-Adeno-X-CMV acceptor plasmid using the Adeno-X Expression System II Kit according to the manufacturer's instructions (Clontech). The plasmids were transformed into *E. coli*, screened for correct recombination, and purified using a Qiagen Endo-Free Maxi Kit (Qiagen, Valencia, CA, USA). The adenoviral plasmid was linearized with PacI DNA restriction enzyme and transfected into HEK293 cells in a 6-cm plate using Lipofectamine 2000 (BD Biosciences, Palo Alto, CA, USA) as described previously [40]. Adenovirus infected cells were harvested after 3 days, amplified once, and the resulting viral lysate was used for subsequent infection experiments. The virus was titered by serial dilution and infection of HEK293 cells in 96-well plates as described in the pSilencer adeno 1.0-CMV system kit manual (Ambion). Viral titer is expressed as infective viral units per milliliter (ivu/mL).

Adenoviral Myostatin shRNA

The construction of adenovirus expressing an shRNA, which targets myostatin, was carried out as follows: the shRNA against mouse myostatin had been identified by our group as previously described, with the shRNA inhibiting more than 95% of myostatin gene expression [37,38,40]. Oligonucleotides corresponding to the shRNA were synthesized, annealed, and ligated into the

pSilencer adeno 1.0 shuttle vector according to the manufacturer's instructions (pSilencer adeno 1.0-CMV system kit). The top annealing oligonucleotide was 5′-TCGAGGATGACGAT TATCA CGCTATTCAAGAGATAGCGTGATAATCGT CATCTTA-3′ and the bottom annealing oligonucleotide was 5′-CTAGTAAGATGACGATT ATCACGCTATCTCTTGAATAGCGTGATAA TCGTCA TCC-3′. The DNA sequence consists of a XhoI DNA restriction site, sense strand, nine nucleotide loop, antisense strand, and SpeI DNA restriction site 5′ to 3′. In addition, an shRNA "randomer," provided with the pSilencer kit and known not to block any mammalian mRNA, was also prepared. The shRNA plasmid constructs were identified by DNA sequencing. The pSilencer Adeno 1.0-Mst shRNA plasmid and adenoviral vector backbone plasmid were linearized with Pac1 and cotransfected in HEK293 cells using the calcium phosphate transfection method. Virus lysate was isolated, amplified, and titered as described above for the myostatin cDNA adenovirus, yielding a virus named AdV-Mst shRNA.

Both the AdV-CMV-Mst375 and the AdV-Mst shRNA constructs were tested for their ability to express or block the expression of myostatin in HEK293 cells by western blot, and later in the TA cells, as described under the Results section.

Animal Treatments

Male Fisher 344 rats (8–11 months old, NIH/NIA colony Harlan Sprague–Dawley, Inc., San Diego, CA, USA) were maintained under controlled temperature and lighting and treated according to the National Institutes of Health (NIH) regulations with an institutionally approved protocol. The rats (N = 5/group) were anesthetized with isofluorane (IsoFLO, Abbott Labs, North Chicago, IL, USA) by inhalation in an induction chamber at a concentration of 2.3% and injected in the penile TA close to the middle of the penis with either saline or 0.5 µg TGFβ1 (Biotech Diagnostic, Laguna Niguel, CA, USA), as previously described [19]. Other similar groups received either TGFβ1 together with a single injection in the tunica of AdV-CMV-Mst375 (2×10^6 ivu), or alternatively, after 5 weeks, saline and AdV-Mst shRNA (2×10^6 ivu). During the penile injection, anesthesia was maintained with a face mask. At 45 days after the initial injection into the TA, the rats were pretreated with heparin (1,000 UI/kg; i.p. 15 minutes before perfusion, Elkins-Sinn, Cherry Hills, NJ, USA), anesthetized with thiopental (50 mg/g, Abbott Labs), and perfused through the left ventricle with saline followed by 10% formalin, and the penises were excised. The skin was denuded, removing the glans and adhering noncrural tissue, the penile shaft was separated from the crura, and a 2- to 3-mm transversal slice was cut around the site of the saline or TGFβ1 injection. The tissues were postfixed or fixed overnight in 10% formalin, washed in PBS, and stored at 4°C in 70% ethanol.

Quantitative Estimations in Tissue Sections

For histochemistry and immunohistochemistry, 5-µm adjacent tissue sections obtained from the human or rat tissues were used for at least one of these procedures: (i) collagen/smooth muscle cells ratio by Masson trichrome (Sigma Diagnostic, St. Louis, MO, USA) [19–25] and (ii) myostatin detection by immunodetection, using a rabbit polyclonal purified IgG antibody generated by our group against a 16-amino acid sequence starting at residue 349 (peptide B) common to human and mouse sequences [31]. For myostatin, the sections were quenched in 0.3% H_20_2–PBS, blocked with goat serum (Vector Laboratories, Burlingame, CA, USA), and incubated overnight at 4°C, with the primary antibody at a 1:500 dilution. This was followed by reaction with biotinylated anti-rabbit IgG (Vector Laboratories) for 30 minutes, followed by the ABC complex (1:100; Vector Laboratories) and 3,3′ diaminobenzidine. The sections were counterstained with hematoxylin. Negative controls omitted the primary antibodies or replaced them with IgG isotype at the same concentration. All slides were dehydrated and mounted with permount.

Cells grown on eight-well chamber slides were fixed in 4% p-formaldehyde, quenched with H_2O_2, blocked with normal goat or horse serum, and incubated with specific antibodies for (i) myostatin, with antibody as above and (ii) ASMA mouse monoclonal antibody in Sigma kit, $^1/_2$ dilution (Sigma Chemical, St. Louis, MO, USA), as a marker for myofibroblasts.

Tissue staining was quantified by quantitative image analysis (QIA) using the ImagePro 4.01 program (Media Cybernetics, Silver Spring, MD, USA) coupled to an Olympus BHS microscope equipped with a Spot RT color digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) [19–25,37–41]. The PD-like plaque in the rat TA was estimated by Masson trichrome staining within the half section of the corpora cavernosa where the tunical injection was given. The plaque size was expressed as the ratio between the

area that stained positive for collagen fibers (blue), divided by the total area of smooth muscle cells (red) plus the remaining lacunar spaces and the cytoplasm of nonstained cells, mainly fibroblasts (white). For myostatin, using a computer-generated grid, the number of positive cells was counted in the TA, and the results were expressed as the number of positive cells per field area, and as the total intensity (optical density) per field area. Five nonoverlapping fields were screened. Six sections per tissue specimen from the groups of eight rats were then used to calculate the mean ± standard error of the mean (SEM).

For nonquantitative dual confocal microscopy, the primary antibodies against myostatin and ASMA were as described above, and the secondary anti-mouse IgG antibody was biotinylated (goat, 1/200, Vector Laboratories). The complex was detected using streptavidin linked to Texas Red (red) or FITC (green). After washing with PBS, the sections were mounted with Prolong antifade (Molecular Probes, Carlsbad, CA, USA). The negative controls in all cases omitted the first antibodies or they were replaced by IgG isotype. Tissue sections or cells were visualized under a Leica TCS SP confocal laser-scanning microscope equipped with argon and HeNe lasers coupled to an acquisition software. Images were imported to Adobe Photoshop 7.0, cropped and adjusted for brightness and contrast only, and saved as TIFF files.

Western Blot and Densitometry Analysis

Cell lysates (20–50 µg of protein) were subjected to western blot analyses [31,37–41] by 4–15% Tris-HCl polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) in running buffer (Tris/glycine/sodium dodecyl sulfate). Proteins were transferred overnight at 4°C to nitrocellulose membranes in transfer buffer (Tris/glycine/methanol). The next day, nonspecific binding was blocked by immersion of the membranes in 5% nonfat dried milk, 0.1% (v/v) Tween 20 in TBS for 1 hour at room temperature. After several washes with washing buffer (TBS Tween 0.1%), the membranes were incubated with the primary antibodies for 1 hour at room temperature. Monoclonal antibodies were as follows: (i) ASMA, monoclonal (1/1,000) (Calbiochem, La Jolla, CA, USA); (ii) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1/10,000) (Chemicon International, Temecula, CA, USA) [41]; and (iii) myostatin, using a mouse monoclonal antibody against the myostatin carboxy-terminal 113 amino acids [38]. In negative controls, we either omitted the first antibody or used a nonimmune IgG. The washed membranes were incubated for 1 hour at room temperature with 1/3,000 dilution of anti-mouse secondary antibody linked to horseradish peroxidase. After several washes, the immunoreactive bands were visualized using the Super Signal West Pico Chemiluminiscent detection system (Pierce, Rockford, IL, USA). The densitometry analysis of the bands was done with the Scion Image software beta 4.0.2 (Scion Corp., Frederick, MD, USA).

RT-PCR and Ribonuclease Protection Assay

Two micrograms of total RNA extracted from human tissues or cultured cells using the Trizol reagent (Invitrogen) were reverse transcribed, and cDNA was amplified for 35 cycles by PCR at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute [37,38,40]. PCR products were analyzed in 2% agarose gels. In some cases, a multiplex reaction was carried out, using the primers ii, iii, v, vi, and vii, in combination. The sequences of the Mst forward/reverse PCR primers, and the predicted fragment sizes, are as follows: (i) myostatin: forward: 5′-GACAAAACACGAGGTACTC, reverse: 5′-TGGATTCAGGCTGTTTGAGC (531 bp); (ii) myostatin: forward: 5′-GGAAA CAA TCATTACCATGC, reverse: 5′-ATCCATAGT TGGGCCTTTAC (129 bp); (iii) ASMA: forward: 5′-CCGGGACATCAAGGAGAAAC, reverse: 5′-CATAGTGGTGCCCCCTGATA (289 bp); (iv) GAPDH: forward: 5′-ATCACTG CCACC CAGAAGACT, reverse: 5′-CATGCC AGTGAGCTTCCCGTT (152 bp); (v) GAPDH: forward: 5′-CATGGGGAAGGTGAAGGTCG, reverse: 5′-TTACTCCTTGGAGGCCATG (1,009 bp); (vi) collagen I-α: forward: 5′-AGGT GCTACATCTATGTGAT, reverse: 5′-TTCCA CATGCTTTATTCC AG (510 bp); and (vii) BMP-2: forward: 5′-TTGGACACCAGGTTGG TGAA, reverse: 5′-AGGCGT TTCCGCTGTT TGT (302 bp).

A ribonuclease protection assay [44] was carried out to conclusively determine the presence of the myostatin mRNA. Twenty micrograms of total RNA was used for hybridization based on the manufacturer's protocol (RPA III assay, Ambion). The myostatin probe (80 nucleotides) was in vitro synthesized and radioactively labeled with ^{32}P-UTP using the MaxiScript in vitro transcription kit (Ambion). The probe was DNase treated and ethanol precipitated. Probe (5×10^4 cpm) was coprecipitated with 20-µg nonhomologous RNA

overnight, redissolved, denatured, and hybridized to cellular RNA overnight at 42°C. After hybridization, unprotected single-stranded RNA was digested with RNase, which was then inactivated; after which, the protected RNA was precipitated, redissolved, and run on a 5% denaturing polyacrylamide gel. Following electrophoresis, the gels were dried onto filter paper and exposed to X-ray film for 72 hours.

Statistics

The data are expressed as the mean (SEM). The normality of the data distributions was established by the Wilk–Shapiro test, and pairs of groups were compared by the *t*-test. Multiple comparisons among groups were analyzed by one-way analysis of variance, followed by post hoc Student–Neuman–Keuls tests. Differences were considered significant at $P < 0.05$.

Results

Myostatin is Expressed in the Normal Human TA and Overexpressed in PD Fibrotic Plaque, and the Latter Expression Occurs at Least in Part in Myofibroblasts

Paraffin-embedded tissue sections of the human PD plaque and the normal TA, adjacent to those sections used for previous studies [19], were subjected to immunohistochemistry for myostatin. Representative micrographs (Figure 1A, B) show that only a few discrete cells were positive in the normal TA (Figure 1A), whereas a much larger number of cells were intensively stained in the plaque (Figure 1B). QIA (Figure 1, bottom) showed a significant increase in both the number of positive cells (over threefold) and in the intensity of staining in the PD plaque as compared with the normal TA.

The immunohistochemical detection of myostatin was validated by identifying the mRNA for myostatin in three specimens of the PD plaque by RT-PCR, with a set of primers spanning two exons, which rules out any contribution from eventual DNA contamination, that leads to a 510-bp fragment (Figure 2A). The RNA from the normal penile corpora cavernosa generated a much fainter band, suggesting that it stems mainly from the tunica. The band in the positive control skeletal muscle tissue was intense, as expected, and was absent in the negative control reaction of PD plaque RNA in which reverse transcriptase was omitted. A second validation, the confirmation of the expression at the protein level, was obtained by western blot of tissue homogenates from the same PD specimens, showing the 32-kDa putative glycosylated dimer of the 110 amino acid processed protein [31], and the 52-kDa monomeric 375 amino acid full-length protein (Figure 2B).

In the dual fluorescence immunodetection of tissue sections adjacent to the ones examined in Figure 1, virtually all myofibroblasts in the PD plaque (identified in red fluorescence by ASMA immunostaining in Figure 2C, panel A) expressed myostatin (identified in green fluorescence, Figure 2C, panel B), as confirmed by the overlay where positive cells are yellowish (Figure 2C, panel C). However, myostatin was also expressed in cells not positive for ASMA, and hence, not identifiable as myofibroblasts, possibly fibroblasts or stem cells [41,45].

Myostatin is Also Overexpressed in Human PD Plaque Cells, Stimulates Myofibroblast Generation and Collagen Expression in Normal TA Cells, Is Up-Regulated and Translocated by TGFβ1, and Potentiates the Effects of TGFβ1

As the cell cultures obtained from both the normal human TA and the PD plaque are mostly fibroblasts but, in addition, contain both myofibroblasts and stem cells [41,45], we investigated, by RT-PCR, the relationship between the myofibroblast content and myostatin expression at different cell passages. Figure 3A shows that little myostatin mRNA was expressed in the normal tunical cells, but this expression increased with passage number, in parallel to a similar increase in the faint ASMA band, denoting simultaneous myofibroblast generation. In contrast, the PD cells had, from the very early period, much higher levels of myostatin than the tunical cells. This expression in the PD cells remained constant with passage, paralleling robust expression of ASMA, in close correspondence to the situation in vivo. These differences between cultures from the PD plaque and the TA would be even more pronounced if the band intensities were normalized for the relatively lower expression in the PD cells of GAPDH.

To exclude any artifact in the detection of myostatin mRNA from the cells, as RT-PCR may pick up even RNA breakdown products or contaminating DNA, we carried out a ribonuclease protection assay to visualize the predicted 225-bp protected fragment that would be a better indication of intact mRNA. Figure 3B shows a clear band in passage 2 from the human PD plaque cells, which however, was very faint in passage 10, suggesting some degradation. The same band was visible in

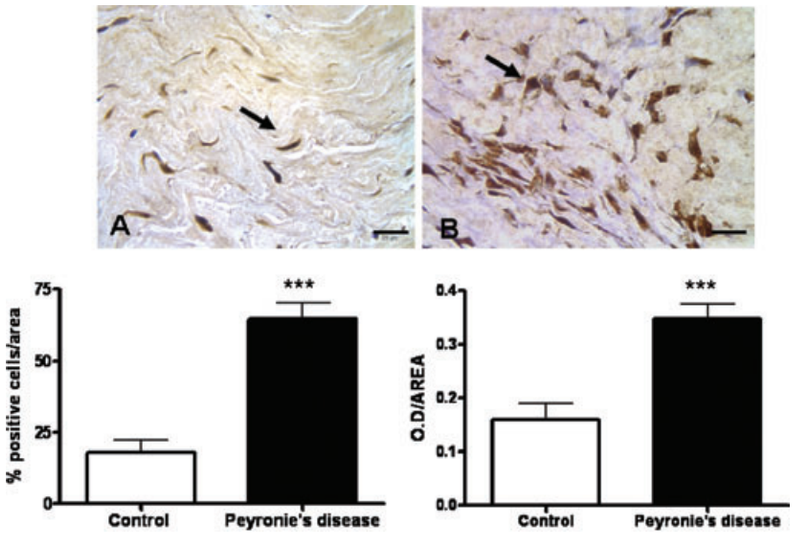


Figure 1 Myostatin is overexpressed in discrete cells in the human Peyronie's disease (PD) plaque, as compared with the normal tunica albuginea. (A, B) Representative micrographs of paraffin-embedded tissue sections stained with myostatin antibody by immunohistochemistry, without counterstain. (A) Tunica albuginea from normal subjects; (B) fibrotic PD plaque. (Bottom) Quantitative image analysis (N = 5/group). ***$P < 0.001$.

the RNA from the corresponding tunical passages, and as expected, in the positive control (the human skeletal muscle), and absent in the negative control, yeast. This unequivocally confirmed the expression of myostatin RNA in both the human TA and PD cells.

As TGFβ1 up-regulates ASMA levels in TA cells [41], implying myofibroblast generation, and as TGFβ1 is a key profibrotic factor expressed in the human PD plaque [19,20] and also an inducer of a PD-like plaque in the rat model [19,20,24,35], we next investigated whether TGFβ1 exerted any effect on myostatin localization and content in the TA and PD cells. Myostatin was mainly localized in the nuclei, as shown under high magnification in Figure 4A, and at lower magnification, that

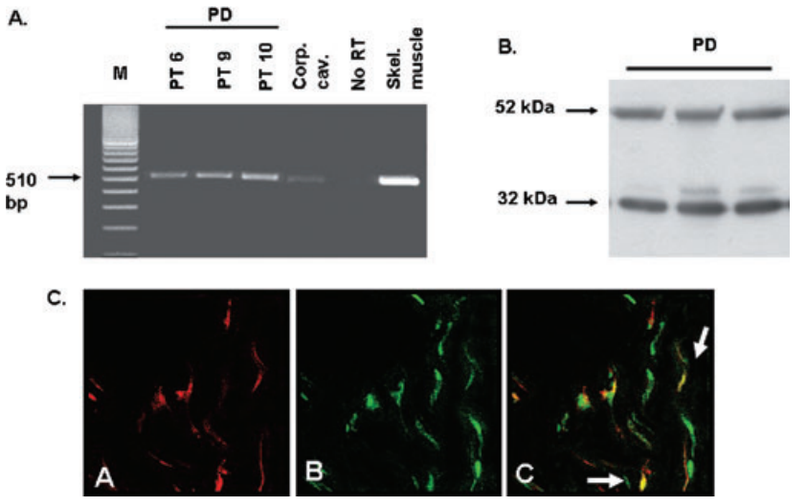


Figure 2 Myostatin expression in the human fibrotic Peyronie's disease (PD) plaque was confirmed by RT-PCR and western blot, and detected in the myofibroblasts by immunofluorescence. (A) RNA was isolated from three specimens of PD plaque, subjected to RT-PCR that yields a 510-bp RNA, and visualized by ethidium bromide staining on agarose gels. (B) Aliquots from the same three specimens of PD plaque were subjected to western blot for myostatin and luminol reaction, identifying two bands of 32 and 52 kDa. (C) Representative micrographs for tissue sections adjacent to those on Figure 1B. The sections were reacted sequentially with α-smooth muscle actin (ASMA) and myostatin primary antibodies, followed by specific secondary antibodies linked to either Texas Red (panel A, ASMA) or biotin and streptavidin-FITC (panel B, myostatin). The sections were examined separately (panels A, B) and after overlay (panel C) under a regular fluorescent microscope. Dual-stained cells are indicated with arrows. Corp. cav. = human corpora cavernosa RNA; no RT = reaction for PT10 RNA conducted without reverse transcriptase; skel. muscle = positive control from human skeletal muscle RNA.

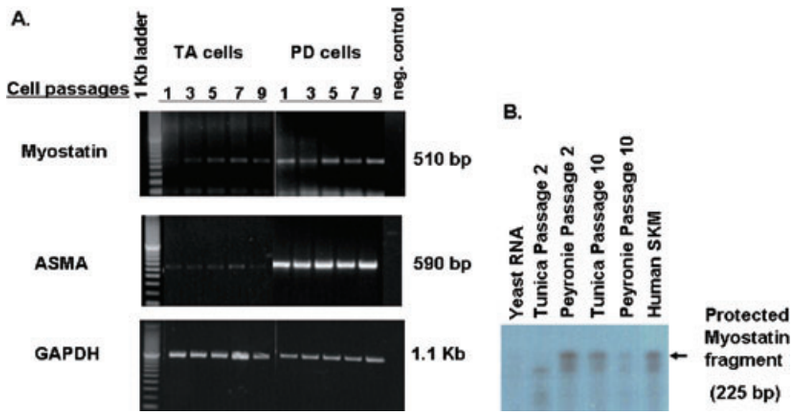


Figure 3 Cell cultures from the human Peyronie's disease (PD) plaque containing myofibroblasts overexpress myostatin, in comparison to cells from the normal tunica albuginea (TA), as determined by RT-PCR and ribonuclease protection assay. (A) RNA was isolated from the cell cultures from the normal TA (TA cells) and PD plaque (PD cells) at passages 1–9, and was subjected to RT-PCR for myostatin as in Figure 2A, for α-smooth muscle actin (ASMA), and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reporter gene. (B) RNAs between passages 2 and 10, and from the yeast (negative control) and skeletal muscle (SKM) (positive control) were subjected to RNA protection assay and ran on a polyacrylamide gel, detecting the 225-bp protected fragment from the mRNA by hybridization to a cDNA probe.

incubation with TGFβ1 partially translocated myostatin to the cytoplasm (Figure 4C), in comparison to untreated cells where cytoplasmic myostatin was low (Figure 4B). To determine whether myostatin expression may be a factor in myofibroblast differentiation, human normal TA cells were incubated for 10 days with recombinant human myostatin proteins corresponding to the full-

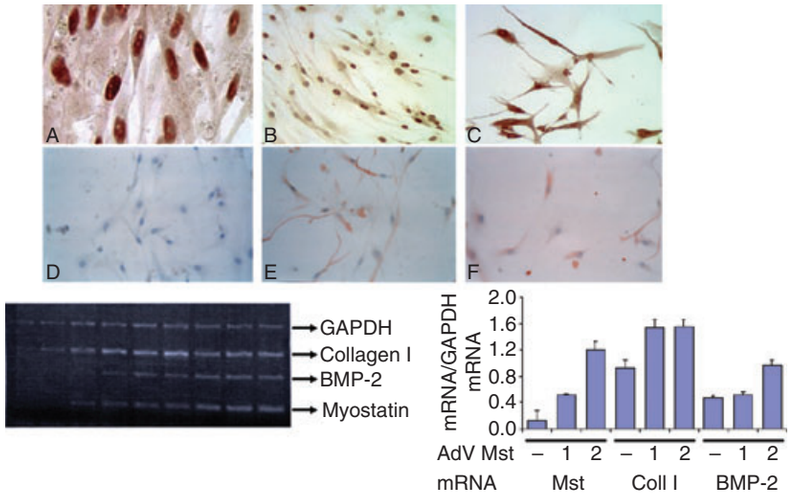
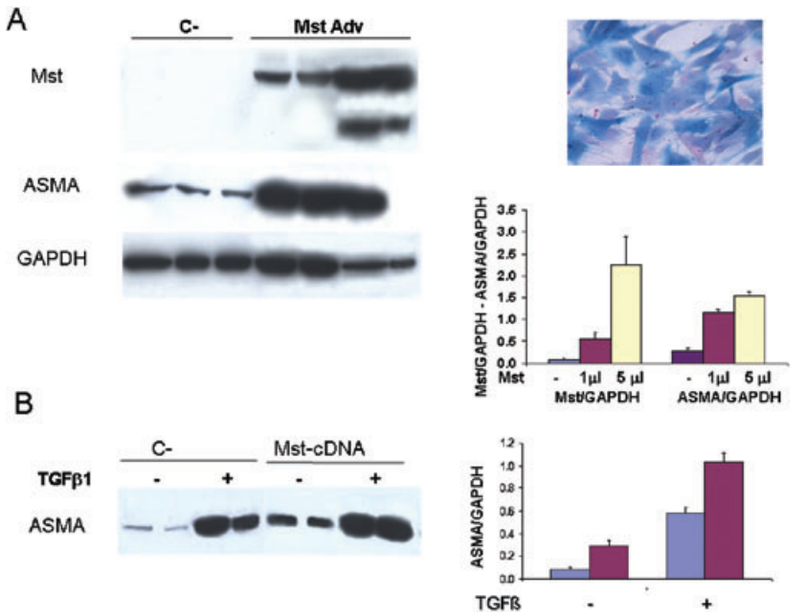


Figure 4 Myostatin mainly localizes in the nuclei of the human Peyronie's disease (PD) plaque cells, translocates upon incubation with the transforming growth factor β1 (TGFβ1), and in the tunica albuginea cells, stimulates α-smooth muscle actin (ASMA) and collagen mRNA expression. (A–C) Representative micrographs of human PD plaque cells were stained with the antibody against myostatin. (A) Control cells (400×); (B) control cells (100×); (C) cells treated with 5 ng/mL TGFβ1 (100×). (D–F) Representative micrographs of human tunica albuginea cells (100×) incubated for 1 week with recombinant human myostatin proteins corresponding to the 110- (F) or the 375-amino acid sequences (E), or without additions (D), and then stained for ASMA. (Bottom left) Ethidium bromide stained agarose gel for multiplex RT-PCR reactions on RNAs from tunical cells incubated for 1 week in special medium, osteogenic medium, that were left untreated or transduced with the adenoviral cDNA construct for Mst375 under the CMV promoter construct at 2 ivu differing by a factor of five. The experiment was carried out in triplicate, as shown. (Bottom right) Densitometric values corrected by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). BMP-2 = bone morphogenic protein-2.



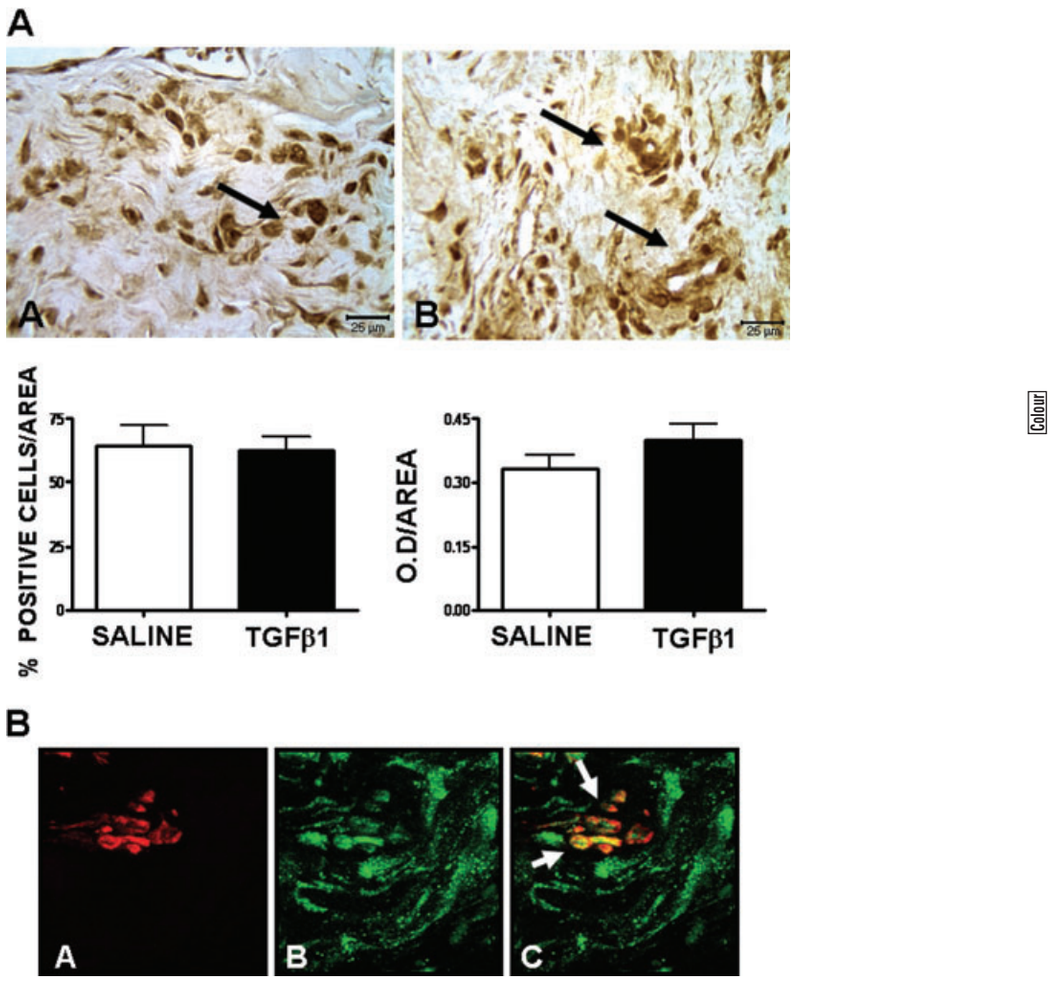
Colour

Figure 5 The stimulation of α-smooth muscle actin (ASMA) mRNA expression by myostatin in tunica albuginea cells is also detectable at the protein level and is additive to the transforming growth factor β1 (TGFβ1) effects. (A, left) Representative western blots for tunica albuginea cells left untreated (C) or transduced with 1 or 5 µL of adenoviral cDNA construct for Mst375 under the CMV promoter (Mst AdV) for 10 days, separately probed with antibodies for myostatin, ASMA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (A, top right) Tunica albuginea cells transduced with the corresponding AdV construct expressing β-galactosidase and assayed after 3 days with X-gal. (A, bottom right) Corresponding densitometric analysis of ASMA (38 kDa) and myostatin (50 kDa) band intensities corrected by GAPDH. (B, left) As in A (left) but with cells treated or not with TGFβ1 (5 ng/mL); (B, bottom right) Corresponding densitometric analysis of myostatin band intensities corrected by GAPDH.

length 375 amino acid (Mst375) (Figure 4E) or the processed carboxy-terminus 110 amino acid (Mst110) (Figure 4F) sequences, or without myostatin (Figure 4D). Both forms of myostatin induced ASMA expression, suggesting that they stimulate myofibroblast generation.

To determine whether myostatin produces a profibrotic effect, we transfected TA cells with an adenoviral construct containing cDNA, encoding the full-length myostatin protein (AdV-CMV-Mst375), and incubated them for 1 week in a medium (OM) that stimulates the fibrogenic and osteogenic differentiation of these cultures [41,43]. Transfected cells were compared with nontransfected controls; RNA was extracted from the cultures and subjected to a simultaneous multiplex RT-PCR analysis for four different genes, using sets of primers that generate DNA fragments that can easily be discriminated by gel electrophoresis (Figure 4, bottom left). In the OM, myostatin RNA expression is virtually negligible, but is significantly expressed after transduction with the myostatin cDNA construct, even at the lower viral load ("1," roughly equivalent to 2–5 ivu). Collagen I-α RNA, a typical end product in the PD plaque, and fibrosis in general, was stimulated in parallel with myostatin. The same result was observed for BMP-2, another TGFβ family member that has not been associated with fibrosis but rather with osteogenesis in general [28] and with PD fibrotic plaque ossification in particular [41]. Densitometric analysis for the expression of each band corrected by GAPDH confirmed this visual evaluation (Figure 4, bottom right).

To confirm the mRNA results at the protein level, normal TA cells were transduced with the AdV-CMV-Mst375 construct and incubated for 10 days. Western blot analysis for myostatin expression shows a substantial dose-dependent overexpression induced by the construct, accompanied by a parallel stimulation of ASMA (Figure 5A). The 100% transduction efficiency of this adenoviral vector was confirmed by X-gal staining of cells transduced in parallel with a construct of the same vector expressing β-galactosidase. To assess whether myostatin effects on ASMA expression are additive to those of TGFβ1, the experiment was repeated in the presence and absence of



Colour

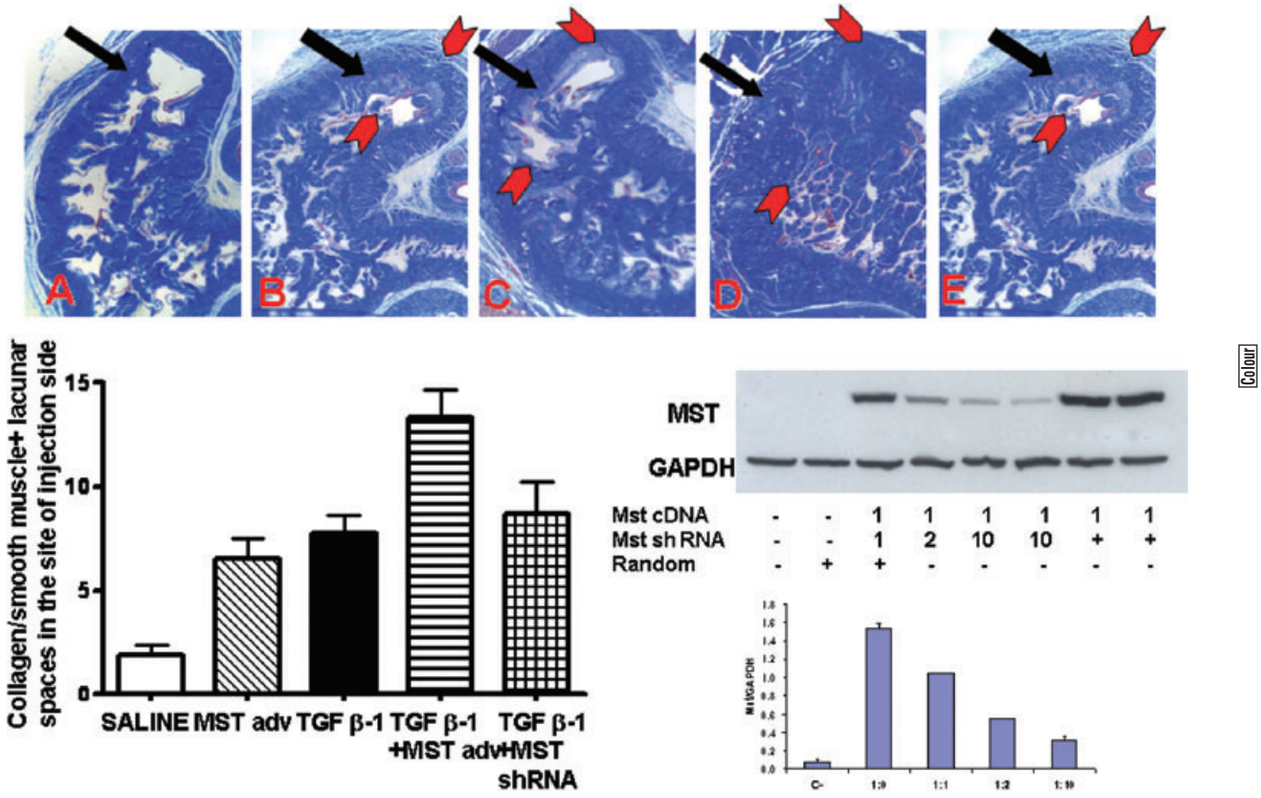
Figure 6 Myostatin is expressed in myofibroblasts in the Peyronie's disease (PD)-like plaque induced by the transforming growth factor β1 (TGFβ1) in the rat tunica albuginea, but in contrast to the human tissue, this does not involve substantial overexpression. (A, top) Representative micrographs (200×, bar = 25 μm) for penile tissue sections around the site of injection from rats treated with either saline (a) or 0.1 μg of TGFβ1 (b) and maintained for 45 days. Immunostaining was performed for myostatin as in Figure 1. (A, bottom) Quantitative image analysis (N = 6/group). (B) Micrographs (200×, bar = 25 μm) for tissue sections adjacent to those in panel A, PD tissue. The sections were stained sequentially by immunofluorescence with α-smooth muscle actin antibody (Texas Red, red fluorescence) and with myostatin antibody (FITC, green fluorescence) as in Figure 2, and examined separately and after overlay under a confocal microscope. Dual-stained cells are indicated with arrows.

37

26 TGFβ1 (5 ng/mL). Figure 5B shows the expected results, namely stimulation of ASMA expression by TGFβ1, potentiated by the adenoviral myostatin construct.

Expression of Myostatin in the Rat TA Is Substantial Despite the Virtual Absence of Myofibroblasts, but Is Not Increased in a TGFβ1-induced PD-like Fibrotic Plaque Where It Is Partially Located in the Myofibroblasts

Myostatin was immunodetected at the site of saline injection in the TA of control rats not injected with TGFβ1. At 45 days after TGFβ1 injection, a surprisingly large level of myostatin expression in discrete cells was observed, nearly as high as in the plaque (Figure 6A, top, a vs. b). QIA (Figure 6A, bottom) confirmed the visual observation. Neither the considerably elevated number of positive cells per unit area in the rat TA (about 60%, well above the 20% in the human TA shown in Figure 1) nor the intensity were significantly different in the TGFβ1-induced plaque. Therefore, endogenous myostatin production in the TA fibroblasts is not by itself sufficient to induce myo-



Colour

Figure 7 Myostatin overexpression in the rat tunica albuginea induced a small Peyronie's disease-like plaque and increased the transforming growth factor β1 (TGFβ1)-induced fibrosis, but myostatin was not necessary for plaque generation. (Top) Representative micrographs of Masson trichrome-stained tissue sections around the site of intratunical injection from the penis of rats treated for 45 days (N = 5/group) as follows: (A) saline; (B) AdV-CMV-Mst cDNA (2×10^6 ivu). (Bottom [left, right]) Quantitative image analysis of these two groups and of similar penile cross sections from additional groups, as follows: (C, top) TGFβ1 (0.5 μg); (D, top) AdV-CMV-Mst cDNA and TGFβ1; and (E, top) AdV-CMV-Mst shRNA (2×10^6 ivu), injected 2 weeks before sacrifice.

38
39

fibroblast formation or to elicit the fibrotic plaque. As in the case of the human PD plaque, myostatin in the rat counterpart is expressed in myofibroblasts, as shown by double immunofluorescence detection of ASMA and myostatin, analogous to the experiment performed on human tissues (Figure 6B) and also in other cells in the rat PD-like plaque previously identified as fibroblasts by vimentin staining [19,41,42].

Myostatin Can Induce or Stimulate Fibrosis in the Rat TA, and the PD-like Plaque Induced by TGFβ1 Is Enhanced but Does Not Seem to be Mediated by Myostatin

The previous experiment showed that considerable endogenous expression of myostatin occurs in the rat TA, even in the absence of plaque formation, whereas in human, it is restricted to the PD plaque itself. To elucidate this apparent discrepancy, we used a more direct approach to determine whether myostatin could induce new plaque development in the rat TA or exacerbate an already preformed plaque. Figure 7 (top) shows the representative micrographs of Masson trichrome staining that detect collagen fibers in blue, and smooth muscle cells in red. The AdV-CMV-Mst375 was injected directly into the TA of the rat, and 45 days later, a PD-like plaque was observable (Figure 7B vs. control in Figure 7A), not significantly different in size from the one induced by TGFβ1 (Figure 7C). When the Adv-Mst construct was injected simultaneously with TGFβ1 (Figure 7D), the plaque was increased in size as compared with the ones generated by TGFβ1 alone. However, the inhibition of myostatin expression with an injection of the corresponding adenoviral anti-myostatin shRNA (AdV-Mst shRNA), that specifically breaks down myostatin mRNA [37,38,40], did not reduce the plaque induced by TGFβ1. This was confirmed by QIA (Figure 7,

bottom left). The plasmid construct expressing myostatin shRNA had previously been shown to inhibit myostatin expression in HEK293 cells, 10T1/2 cells, rat cardiomyocytes, and in skeletal muscle [37,38,40]. We confirmed this effect in TA cells by incubation for 3 days with AdV-CMV-Mst375 either alone or in the presence of increasing ratios of AdV-Mst shRNA (Figure 7, bottom right), which showed that the shRNA construct dose dependently inhibited myostatin expression.

Discussion

These results are the first demonstration of the expression of a second TGFβ family member, myostatin, in addition to TGFβ1 itself, in the normal human penile TA and in the human PD fibrotic plaque. In the latter tissue, there is an overexpression of myostatin and this corresponds to what is seen in the cell cultures of the normal human TA and PD plaque. A similar effect was also seen in the corresponding tissues from the experimental rat model of PD, although in the rat tunica, the expression is as high as in the PD-like lesion. The latter tissue resembles the human plaque in the accumulation and disorganization of collagen fibers, appearance of myofibroblasts, oxidative stress and expression of fibrotic markers, as well as by the induction of the antifibrotic factor inducible nitric oxide synthase. Fibrosis in this rat model is not normally accompanied by chronic inflammation [13,15,18–22]. Although the levels of myostatin in the normal rat tunical fibroblasts are considerable, they are not sufficient to induce myofibroblast formation or fibrosis, and therefore, other ancillary factors may be required for such effects to occur.

Myostatin is expressed both in vivo and in vitro in myofibroblasts, the cells that play a major role in fibrosis. They are characterized by vimentin and ASMA expression, and although the latter marker is also present in smooth muscle cells, their presence was excluded by the absence of smoothelin when the primary cultures were obtained [20,41,42]. In tissue sections, myofibroblasts are easily differentiated from smooth muscle cells by their respective restricted location in the TA and the corpora cavernosa [19,20]. Myostatin expression is seen in the human plaque and in the rat lesion induced by TGFβ1, and in vitro, myostatin triggers the generation of myofibroblasts, presumably from stem cells present in the tunical fibroblast cultures [41,45]. The profibrotic action of myostatin is supported by the finding that in vivo, it can elicit a PD-like plaque in the rat TA and intensify the one triggered by TGFβ1, although the effects of TGFβ1, similar to what occurs in the TA cell cultures, do not seem to require myostatin.

Collectively, the current results extend previous in vitro findings on the intensification by myostatin of the fibrotic phenotype of myofibroblasts derived from the multipotent C3H 10T1/2 cell line [36,37], as well as on the cell lineage differentiation of skeletal muscle fibroblasts [36]. Therefore, myostatin joins not just TGFβ1 but other TGFβ family members such as activin and possibly, BMP-4 [26,28], which have been acknowledged as profibrotic factors, and suggests that antifibrotic therapies aimed at inhibiting common converging downstream effectors for all these proteins would be more effective than trying to block single agents within the family individually.

Myostatin was initially thought to be restricted to skeletal muscle and cultured myotubes [30,31], but subsequently, it was also found to be expressed at low levels in cardiomyocytes [46], differentiating multipotent C3H 10T1/2 cells [37,38], and fibroblasts [36]. This suggests that the role of myostatin was not restricted just to being a negative regulator of skeletal muscle mass, but, in agreement with its original designation as GDF-8, myostatin may also participate in cardiomyocyte, adipocyte, and myofibroblast differentiation [36–38,46]. In contrast to the well-characterized inhibitory action of myostatin on myogenesis, its effects on cardiomyogenesis and adipogenesis are still controversial, but the induction of myofibroblasts and of fibrotic factors by myostatin has been confirmed by at least two groups [36,37]. In an article directly relevant to myofibroblast differentiation, it was shown that myostatin induced in vitro myofibroblast generation from skeletal muscle fibroblasts present in the so-called "PP1" fraction during the MDSC PP6 isolation, as indicated by ASMA levels, and that this was accompanied by the deposition of collagen I and III [36].

Our current results in the normal human TA fibroblasts indicate that these profibrotic effects of myostatin occur in fibroblasts from at least two sources. However, in the human TA cell cultures and rat TA tissue, stem cells have been identified [41,45], and it is not yet clear whether they or the true fibroblasts are the cells undergoing myofibroblast differentiation. We have shown here that myostatin is expressed in TA myofibroblasts and that in vitro, this occurs in the nuclei, in agreement with what has been shown in skeletal muscle fibroblast cultures. This has also been found in another

multipotent cell line of fibroblast origin, the mouse C3H 10T1/2 cells, despite the membrane location of its receptor, the activin IIb receptor [46]. Interestingly, the de novo myofibroblast generation induced by myostatin that we have observed was not found in the C3H 10T1/2 cells. Although myostatin is profibrotic in the myofibroblasts themselves, as shown by its effect of switching them to the synthetic phenotype, it does not stimulate stem cell/myofibroblast lineage differentiation per se [37]. The fact that TGFβ1 stimulates myostatin expression, and conversely that myostatin up-regulates TGFβ1 levels, was shown in the case of skeletal muscle fibroblasts [36], and the same observation was also found with the C3H 10T1/2 cells [37]. This mutual interaction between both profibrotic members of the TGFβ family has been confirmed here with TA/PD fibroblast cultures that are known to contain stem cells [41].

It is intriguing that myostatin could induce myofibroblast formation in vitro and a PD-like fibrotic plaque in the rat TA, and stimulate the in vivo plaque induction by TGFβ1, whereas blocking myostatin expression by the shRNA against myostatin did not inhibit plaque formation induced by TGFβ1, and also, endogenous high myostatin expression in the normal rat TA was not associated with fibrosis. It is therefore likely that the myostatin profibrotic effects may proceed mainly via endogenous TGFβ1 induction, as in the case of fibrin [29,30], whereas exogenous TGFβ1 does not seem to require myostatin to act. In fact, decorin, an antifibrotic proteoglycan that binds to and blocks the activity of TGFβ1, neutralizes the effects of myostatin on the fibroblast generation of myofibroblasts [36,47]. However, both decorin [36] and myostatin itself [37] up-regulate the expression of follistatin, an antagonist of these two members of the TGFβ family [48] and also an antagonist to a third member, activin; so, it is difficult to determine whether myostatin can act as a profibrotic agent independently from TGFβ1 secretion. Further studies need to be conducted using a specific blocker of TGFβ1 activity that does not affect myostatin, such as shRNA against TGFβ1.

In earlier experiments, the shRNA construct against myostatin blocked its expression both in vivo in the skeletal muscle [40] and in vitro in several cell cultures [37,38]. In the current study, it was shown to block myostatin expression in tunical cells, but this inhibition did not reduce the PD-like plaque in the rat model. We cannot discard the possibility that the shRNA did not efficiently inhibit myostatin expression in the PD-like plaque, but even if it did, this does not necessarily mean that the profibrotic effects of myostatin are unimportant in the development of the human condition. First, it is quite likely that, as in the skeletal muscle myofibroblasts, there may be a synergistic effect of myostatin and TGFβ1. Second, it is still possible that the TGFβ1-induced plaque in the rat TA does not mimic in this respect the human PD plaque, where TGFβ1 levels may be much lower. Therefore, myostatin would act in that latter setting as a true cofactor in fibrotic induction.

Although anti-TGFβ1 therapeutic strategies have not been tested for PD either experimentally (because the rat lesion requires this agent for the development of PD) or clinically, the potential for intervention against myostatin and other TGFβ family members based on the effects seen in this study, suggests that it would be better to collectively inhibit all these agents rather than focus on inhibiting just one. In this sense, both follistatin and decorin, which have been tested successfully in skeletal muscle fibrosis [47,49], may be worth investigating in rat models of PD, in particular, the model in which the plaque is elicited by fibrin instead of TGFβ1 itself, even if this latter factor is also induced in this type of lesion [24].

Another potential target is the Smad gene set. This acts through a downstream pathway common to TGFβ1, myostatin, and activin, thereby transducing the profibrotic effects of these proteins [27,29]. In this sense, cDNA constructs of the inhibitory Smad7 [50] may be used as a proof of concept, as well as inhibitors of cAMP-dependent PDEs such as forskolin or isoproterenol. The latter agents, via the increase of cAMP inhibit TGFβ1-stimulated collagen synthesis and ASMA expression in cardiac fibroblast cultures, in part, by reducing binding of the transcriptional coactivator CREB-binding protein 1 to transcriptional complexes containing Smad2, Smad3, and Smad4 [51]. It appears that cAMP-elevating agents inhibit the profibrotic effects of TGFβ1, partially by reducing the Smad-mediated recruitment of transcriptional coactivators. This would explain our previous results where we observed that a nonspecific PDE inhibitor, pentoxifylline, blocked the development of the TGFβ1-induced fibrotic plaque in the rat TA [22].

On the other hand, BMP-7, a well-known antifibrotic member of the TGFβ family, acting on liver and kidney [52,53], counteracts myofibroblast

differentiation and collagen deposition triggered by TGFβ1. This happens in part by inhibition of the expression of its effector, Smad3 [54]. Myostatin increased BMP-7 and Smad7 expression in the multipotent C3H 10T1/2 cells [37]. Independent of the Smad pathway, the relationship of myostatin with the CTGF, an effector for TGFβ1 fibrotic signaling that in part signals through Smad by inhibiting Smad7 and promoting Smad2 [55], needs to be clarified. In conclusion, the multiplicity of TGFβ members that may act as profibrotic agents (particularly TGFβ1 and myostatin) or antifibrotic (BMP-7), their mutual regulatory interaction, their putative synergistic action, and their common signaling pathways should stimulate further studies to clarify their relative contribution to myofibroblast generation and fibrosis development in general, and specifically in PD. This may help to develop novel therapeutic targets for these conditions.

Acknowledgments

This work was supported by grants NIH R01DK-53069, Department of Defense PR064756, and in certain aspects, NIH G12RR-03026. Dr. Ferrini is also supported by Grant N1 P20 MD000545 from the National Center on Minority Health and Health Disparities, NIH.

Corresponding Author: Nestor F. Gonzalez-Cadavid, PhD, LABioMed at Harbor-UCLA Medical Center, Urology, Bldg. F-6, 1124 West Carson Street, Torrance, CA 90502, USA. Tel: (310) 222-3824; Fax: (310) 222-1914; E-mail: ncadavid@ucla.edu

Conflict of Interest: None declared.

Statement of Authorship

Category 1

(a) Conception and Design
Nestor F. Gonzalez-Cadavid

(b) Acquisition of Data
Liliana P. Cantini; Monica G. Ferrini; Dolores Vernet; Thomas R. Magee; Ansha Qian; Robert A. Gelfand

(c) Analysis and Interpretation of Data
Nestor F. Gonzalez-Cadavid; Monica G. Ferrini; Liliana P. Cantini; Dolores Vernet; Thomas R. Magee; Ansha Qian; Robert A. Gelfand

Category 2

(a) Drafting the Article
Nestor F. Gonzalez-Cadavid; Jacob Rajfer; Monica G. Ferrini

(b) Revising It for Intellectual Content
Nestor F. Gonzalez-Cadavid; Jacob Rajfer; Monica G. Ferrini

Category 3

(a) Final Approval of the Completed Article
Nestor F. Gonzalez-Cadavid

References

1 Schwartz EJ, Wong P, Graydon RJ. Sildenafil preserves intracorporeal smooth muscle after radical retropubic prostatectomy. J Urol 2004;171:771–4.
2 Iacono F, Giannella R, Somma P, Manno G, Fusco F, Mirone V. Histological alterations in cavernous tissue after radical prostatectomy. J Urol 2005; 173:1673–6.
3 Nandipati KC, Raina R, Agarwal A, Zippe CD. Erectile dysfunction following radical retropubic prostatectomy: Epidemiology, pathophysiology and pharmacological management. Drugs Aging 2006;23:101–17.
4 Ostojic P, Damjanov N. The impact of depression, microvasculopathy, and fibrosis on development of erectile dysfunction in men with systemic sclerosis. Clin Rheumatol 2007;26:1671–4.
5 Kovanecz I, Rambhatla A, Ferrini M, Vernet D, Sanchez S, Rajfer J, Gonzalez-Cadavid N. Long-term continuous sildenafil treatment ameliorates corporal veno-occlusive dysfunction (CVOD) induced by cavernosal nerve resection in rats. Int J Impot Res 2007 [Epub ahead of print].
6 Burnett AL. Molecular pharmacotherapeutic targeting of PDE5 for preservation of penile health. J Androl 2007;••:••–••. 31
7 Collado A, Batista E, Gelabert-Mas A, Corominas JM, Arano P, Villavicencio H. Detrusor quantitative morphometry in obstructed males and controls. J Urol 2006;176:2722–8.
8 Tuygun C, Imamoglu A, Keyik B, Alisir I, Yorubulut M. Significance of fibrosis around and/or at external urinary sphincter on pelvic magnetic resonance imaging in patients with postprostatectomy incontinence. Urology 2006;68:1308–12.
9 Bercovich E, Barabino G, Pirozzi-Farina F, Deriu M. A multivariate analysis of lower urinary tract ageing and urinary symptoms: The role of fibrosis. Arch Ital Urol Androl 1999;71:287–92.
10 Liu Y. Renal fibrosis: New insights into the pathogenesis and therapeutics. Kidney Int 2006;69:213–7.
11 Eberhardt W, Pfeilschifter J. Nitric oxide and vascular remodeling: Spotlight on the kidney. Kidney Int Suppl 2007;S9–16.
12 Bella AJ, Perelman MA, Brant WO, Lue TF. Peyronie's disease (CME). J Sex Med 2007;4:1527–38.
13 Gonzalez-Cadavid NF, Rajfer J. Mechanisms of disease: New insights into the cellular and molecular pathology of Peyronie's disease. Nat Clin Pract Urol 2005;2:291–8.

14 Ruiz-Ortega M, Rodriguez-Vita J, Sanchez-Lopez E, Carvajal G, Egido J. TGF-beta signaling in vascular fibrosis. Cardiovasc Res 2007;74:196–206.
15 El-Sakka AI, Hassoba HM, Pillarisetty RJ, Dahiya R, Lue TF. Peyronie's disease is associated with an increase in transforming growth factor-beta protein expression. J Urol 1997;158:1391–4.
16 Darby IA, Hewitson TD. Fibroblast differentiation in wound healing and fibrosis. Int Rev Cytol 2007;257:143–79.
17 Henderson NC, Iredale JP. Liver fibrosis: Cellular mechanisms of progression and resolution. Clin Sci (Lond) 2007;112:265–80.
18 El-Sakka AI, Hassan MU, Nunes L, Bhatnagar RS, Yen TS, Lue TF. Histological and ultrastructural alterations in an animal model of PD disease. Br J Urol 1998;81:445–52.
19 Ferrini MG, Vernet D, Magee TR, Shahed A, Qian A, Rajfer J, Gonzalez-Cadavid NF. Antifibrotic role of inducible nitric oxide synthase. Nitric Oxide 2002;6:283–94.
20 Vernet D, Ferrini MG, Valente EG, Magee TR, Bou-Gharios G, Rajfer J, Gonzalez-Cadavid NF. Effect of nitric oxide on the differentiation of fibroblasts into myofibroblasts in the Peyronie's disease fibrotic plaque and in its rat model. Nitric Oxide 2002;7:262–76.
21 Ferrini MG, Kovanecz I, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. Effects of long-term vardenafil treatment on the development of fibrotic plaques in a rat model of Peyronie's disease. BJU Int 2006;97:625–33.
22 Valente EG, Vernet D, Ferrini MG, Qian A, Rajfer J, Gonzalez-Cadavid NF. L-arginine and phosphodiesterase (PDE) inhibitors counteract fibrosis in the Peyronie's disease fibrotic plaque and related fibroblast cultures. Nitric Oxide 2003;9:229–44.
23 Davila HH, Magee TR, Zuniga FI, Rajfer J, Gonzalez-Cadavid NF. Peyronie's disease associated with increase in plasminogen activator inhibitor in fibrotic plaque. Urology 2005;65:645–8.
24 Davila HH, Ferrini MG, Rajfer J, Gonzalez-Cadavid NF. Fibrin as an inducer of fibrosis in the tunica albuginea of the rat: A new animal model of Peyronie's disease. BJU Int 2003;91:830–8.
25 Davila HH, Magee TR, Vernet D, Rajfer J, Gonzalez-Cadavid NF. Gene transfer of inducible nitric oxide synthase complementary DNA regresses the fibrotic plaque in an animal model of Peyronie's disease. Biol Reprod 2004;71:1568–77.
26 Tsuchida K. Activins, myostatin and related TGF-beta family members as novel therapeutic targets for endocrine, metabolic and immune disorders. Curr Drug Targets Immune Endocr Metabol Disord 2004;4:157–66.
27 Wang W, Koka V, Lan HY. Transforming growth factor-beta and Smad signalling in kidney diseases. Nephrology (Carlton) 2005;10:48–56.
28 Fan J, Shen H, Sun Y, Li P, Burczynski F, Namaka M, Gong Y. Bone morphogenetic protein 4 mediates bile duct ligation induced liver fibrosis through activation of Smad1 and ERK1/2 in rat hepatic stellate cells. J Cell Physiol 2006;207:499–505.
29 Parsons CJ, Takashima M, Rippe RA. Molecular mechanisms of hepatic fibrogenesis. J Gastroenterol Hepatol 2007;22(1 suppl):S79–84.
30 Lee SJ. Regulation of muscle mass by myostatin. Annu Rev Cell Dev Biol 2004;20:61–86.
31 Gonzalez-Cadavid NF, Taylor WE, Yarasheski K, Sinha-Hikim I, Ma K, Ezzat S, Shen R, Lalani R, Asa S, Mamita M, Nair G, Arver S, Bhasin S. Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting. Proc Natl Acad Sci USA 1998; 95:14938–43.
32 Gonzalez-Cadavid NF, Bhasin S. Role of myostatin in metabolism. Curr Opin Clin Nutr Metab Care 2004;7:451–7.
33 Wagner KR, McPherron AC, Winik N, Lee SJ. Loss of myostatin attenuates severity of muscular dystrophy in mdx mice. Ann Neurol 2002;52:832–6.
34 Bogdanovich S, Krag TO, Barton ER, Morris LD, Whittemore LA, Ahima RS, Khurana TS. Functional improvement of dystrophic muscle by myostatin blockade. Nature 2002;420:418–21.
35 McCroskery S, Thomas M, Platt L, Hennebry A, Nishimura T, McLeay L, Sharma M, Kambadur R. Improved muscle healing through enhanced regeneration and reduced fibrosis in myostatin-null mice. J Cell Sci 2005;118:3531–41.
36 Zhu J, Li Y, Shen W, Qiao C, Ambrosio F, Lavasani M, Nozaki M, Branca MF, Huard J. Relationships between transforming growth factor-beta1, myostatin, and decorin: Implications for skeletal muscle fibrosis. J Biol Chem 2007;282:25852–63.
37 Artaza J, Singh R, Ferrini MG, Braga M, Tsao J, Gonzalez-Cadavid NF. Myostatin promotes a fibrotic phenotypic switch in multipotent C3H 10T1/2 cells without affecting their differentiation into myofibroblasts. J Endocrinol 2007 (in press). 32
38 Artaza JN, Bhasin S, Magee TR, Reisz-Porszasz S, Shen R, Groome NP, Meerasahib MF, Gonzalez-Cadavid NF. Myostatin inhibits myogenesis and promotes adipogenesis in C3H 10T(1/2) mesenchymal multipotent cells. Endocrinology 2005;146: 3547–57.
39 Taylor WE, Bhasin S, Artaza J, Byhower F, Azam M, Willard DH Jr, Kull FC Jr, Gonzalez-Cadavid N. Myostatin inhibits cell proliferation and protein synthesis in C2C12 muscle cells. Am J Physiol Endocrinol Metab 2001;280:E221–8.
40 Magee TR, Artaza JN, Ferrini MG, Zuniga FI, Cantini L, Reisz-Porszasz S, Rajfer J, Gonzalez-Cadavid NF. Myostatin short interfering hairpin RNA gene transfer increases skeletal muscle mass. J Gene Med 2006;9:1171–81.

41 Vernet D, Nolazco G, Cantini L, Magee TR, Qian A, Rajfer J, Gonzalez-Cadavid NF. Evidence that osteogenic progenitor cells in the human tunica albuginea may originate from stem cells: Implications for Peyronie's disease. Biol Reprod 2005; 73:1199–210.

42 Qian A, Meals RA, Rajfer J, Gonzalez-Cadavid NF. Comparison of gene expression profiles between Peyronie's disease and Dupuytren's contracture. Urology 2004;64:399–404.

43 Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng 2001;7:211–28.

44 Calzone FJ, Britten RJ, Davidson EH. Mapping of gene transcripts by nuclease protection assays and cDNA primer extension. Methods Enzymol 1987;152:611–32.

45 Nolazco G, Kovanecz I, Vernet D, Ferrini M, Gelfand B, Tsao J, Magee T, Rajfer J, Gonzalez-Cadavid NF. Effect of muscle derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. BJU Int 2007 [33] (in press).

46 Artaza JN, Reisz-Porszasz S, Dow JS, Kloner RA, Tsao J, Bhasin S, Gonzalez-Cadavid NF. Alterations in myostatin expression are associated with changes in cardiac left ventricular mass but not ejection fraction in the mouse. J Endocrinol 2007;194:63–76.

47 Li Y, Foster W, Deasy BM, Chan Y, Prisk V, Tang Y, Cummins J, Huard J. Transforming growth factor-beta1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: A key event in muscle fibrogenesis. Am J Pathol 2004;164:1007–19.

48 Amthor H, Nicholas G, McKinnell I, Kemp CF, Sharma M, Kambadur R, Patel K. Follistatin complexes myostatin and antagonises myostatin-mediated inhibition of myogenesis. Dev Biol 2004; 270:19–30.

49 Aoki F, Kurabayashi M, Hasegawa Y, Kojima I. Attenuation of bleomycin-induced pulmonary fibrosis by follistatin. Am J Respir Crit Care Med 2005;172:713–20.

50 Yamanaka O, Ikeda K, Saika S, Miyazaki K, Ooshima A, Ohnishi Y. Gene transfer of Smad7 modulates injury-induced conjunctival wound healing in mice. Mol Vis 2006;12:841–51.

51 Liu X, Sun SQ, Hassid A, Ostrom RS. cAMP inhibits transforming growth factor-beta-stimulated collagen synthesis via inhibition of extracellular signal-regulated kinase 1/2 and Smad signaling in cardiac fibroblasts. Mol Pharmacol 2006;70:1992–2003.

52 Gressner OA, Weiskirchen R, Gressner AM. Evolving concepts of liver fibrogenesis provide new diagnostic and therapeutic options. Comp Hepatol 2007;6:7.

53 Sugimoto H, Grahovac G, Zeisberg M, Kalluri R. Renal fibrosis and glomerulosclerosis in a new mouse model of diabetic nephropathy and its regression by bone morphogenic protein-7 and advanced glycation end product inhibitors. Diabetes 2007;56:1825–33.

54 Izumi N, Mizuguchi S, Inagaki Y, Saika S, Kawada N, Nakajima Y, Inoue K, Suehiro S, Friedman SL, Ikeda K. BMP-7 opposes TGF-beta1-mediated collagen induction in mouse pulmonary myofibroblasts through Id2. Am J Physiol Lung Cell Mol Physiol 2006;290:L120–6.

55 Qi W, Chen X, Twigg S, Zhang Y, Gilbert RE, Kelly DJ, Pollock CA. The differential regulation of Smad7 in kidney tubule cells by connective tissue growth factor and transforming growth factor-beta1. Nephrology (Carlton) 2007;12:267–74.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME GONZALEZ-CADAVID, NESTOR F	POSITION TITLE Professor
eRA COMMONS USER NAME (credential, e.g., agency login) NESTORGON	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Buenos Aires, Argentina	M.Sc.	1961	Biochemistry
University of Buenos Aires, Argentina	Ph.D.	1964	Biochemistry
University of London, England	Ph.D.	1967	Biochemistry

A. Positions and Honors.

1961 Gold medal to the best MSc graduate, University of Buenos Aires
1961-62 Fellowship, National Council of Scientific Research (Argentina)
1964 Gold Medal to the best doctorate dissertation, University of Buenos Aires
1964-66 Fellowship, Natl Council Sci Res (Argentina), work at the Courtauld Inst Biochemistry, London Univ.
1967 Fellowship, WelcomeTrust (England), ibid
1968-71 Assoc. Professor, Dept. Biochemistry., Sch. of Science, Central University, Caracas, Venezuela
1971-92 Full Professor, Dept Cell Biology, School of Science, Central University, Caracas, Venezuela
1978-79 Gosney Visit. Assoc. in Biology, California Institute of Technology, Biology Division, Pasadena, CA
1980 Senior Fellowship, Guggenheim Foundation, Cal. Inst. of Technology, Biology Div., Pasadena, CA
1982 Visiting Professor, University of Buenos Aires, School of Biochemistry, Buenos Aires, Argentina
1984 Fellowship, Internatl Union Against Cancer, City of Hope Med Center, Div Biology, Duarte (CA).
1987-88 Visiting Professor, UCLA School of Medicine, Div of Hematology/Oncology, Los Angeles, CA
1987 E. Roosevelt fellowship, Internatl Union Against Cancer, UCLA Med School, Dept Medicine, Los Angeles (CA); Senior Fellowship, United Nations Univ. ibid
1990-92 National Research Service Award (Senior Fellowship), Popul. Res. Center, Harbor/UCLA Med. Ctr.
1990-96 Adj. Associate Professor, Dept of Surgery/Urology, UCLA School of Medicine, Director Urology Research Laboratory, Harbor-UCLA REI
1996-on Adjunct Professor, Department of Urology, UCLA School of Medicine, and Director, as above
1997-on Professor, Dept of Internal Medicine/Endocrinology, Charles R. Drew University.
2001-on Director, RCMI Molecular Medicine Core, Charles R. Drew University

B. Professional membership

1965-75 Bioch Soc (England); 1987-90 Tissue Cult Assoc (USA); 1989 Amer Assoc for Cancer Res.; 1992-Am Soc Andrology (USA): 1998-Endocrinol Society (USA); 2000 Am Urological Assoc (USA); 2000 Soc Study Impotence; 2000 Soc Study Reproduction; 2004 Sexual Medicine Soc of N Am

C. Selected peer-reviewed publications from 2003-2007 (from a list of 154 on CV)

Ferrini MG, Magee TR, Vernet D, Rajfer J, **Gonzalez-Cadavid NF** (2003) Penile neuronal nitric oxide synthase (PnNOS) and its regulatory proteins are present in hypothalamic and spinal cord regions involved in the control of penile erection. J Compar Neurol 458:46-61

Magee T, Zeller CB, Ferrini M, Davila H, Vernet D, Burnett AL, Rajfer J, **González-Cadavid NF** (2003) A protein inhibitor of NOS (PIN) is expressed in the rat and mouse penile nerves and co-localizes with penile neuronal NOS (PnNOS) Biol Reprod 68:478-488.

Berman JR, Almeida FG, Jolin J, Raz S, Chaudhuri G, **Gonzalez-Cadavid NF** (2003) Androgen receptors, aromatase, and 5--reductase in human vagina: correlation with menopausal status. Fertil Steril, 79:925-931.

Davila H, Ferrini M, Rajfer J, **Gonzalez-Cadavid NF** (2003) Fibrin induction of a Peyronie's-like plaque in the rat penile tunica albuginea. A new model for Peyronie's disease. Br J Urol, 91:830-838.
Reisz-Porszasz S, Bhasin S, Artaza JN, Shen R, Sinha-Hikim I, Houge A, **Gonzalez-Cadavid NF** (2003) Reduction of skeletal muscle mass in a transgenic mouse that hyperexpresses myostatin in the muscle. Am J Physiol, Am J Physiol, 2003 Oct;285(4):E876-888.
Ma K, Mallidis C, Bhasin S, Mahabadi V, Artaza J, **Gonzalez-Cadavid N**, Arias J, Salehian B (2003) Glucocorticoid-Induced Skeletal Muscle Atrophy is Associated with Upregulation of Myostatin Gene Expression. Am J Physiol, 285:E363-371.
Valente EG, Vernet D, Ferrini MG, Qian A, Rajfer J, **Gonzalez-Cadavid NF** (2003) PDE L-arginine and PDE inhibitors counteract fibrosis in Peyronie's plaque and related fibroblast cultures. Nitric Oxide, 9:229-244.
Singh R, Artaza JN, Taylor WE, (**Gonzalez-Cadavid NF***, Bhasin S*; *: equal contributors) (2003) Androgens stimulate myogenenic differentiation and inhibit adipogenesis in C3H 10T1/2 pluripotent stem cells through an androgen receptor-mediated pathway. Endocrinology, 144:5081-508
Liu W, Thomas SG, Asa SL, **Gonzalez-Cadavid NF**, Bhasin S, Ezzat S (2003) Myostatin is a Skeletal Muscle Target of Growth Hormone Anabolic Action. J Clin Endocrin Metabol, 88:5490-5496
Bhasin S, Taylor W, Singh R, Artaza J, Sinha-hickim I, Jasuja R, Choi H, **Gonzalez-Cadavid NF** (2003) The mechanisms of androgen effects on body composition: mesenchymal pluripotent cell as the target of androgen action. J Gerontol, 58:M1103-10.
Ferrini MG, Davila H, Valente EG**, (Gonzalez-Cadavid NF***, Rajfer J*, *equal contributors) (2004) Aging-related induction of inducible nitric oxide synthase (iNOS) is vasculo-protective in the arterial media. Cardiovascular Res ;61:796-805.
Qian A, Meals R, Rajfer J, **Gonzalez-Cadavid NF** (2004) Comparison of gene expression profiles between Peyronie's disease and Dupuytren's contracture. Urology, 64:399-404.
Gonzalez-Cadavid NF, Bhasin S (2004) Role of myostatin in metabolism. Curr Opin Clin Nutr Metabol Care, 7:451-457.
Gonzalez-Cadavid NF, Rajfer J (2004). Therapy of erectile function: Potential future treatments. Endocrine, 23:167-176.
Gonzalez-Cadavid NF, Rajfer J (2004) Molecular and cellular aspects of the pathophysiology of Peyronie's disease. Drug Discovery Today. Disease mechanisms, 1:99-106
Davila HH, Magee TR, Rajfer J, **Gonzalez-Cadavid NF** (2004) Gene therapy with the inducible nitric oxide synthase (iNOS) cDNA regresses the fibrotic plaque in an animal model of Peyronie's disease**.** Biol Reprod, 71:1568-1577
Davila HH, Rajfer J, **Gonzalez-Cadavid NF** (2004) Corporal veno-occlusive dysfunction in the aging rat. evaluation by cavernosometry and cavernosography. Urology, 64:1261-1266.
Gonzalez-Cadavid NF, Rajfer J (2004) Molecular pathophysiology and gene therapy of aging-related erectile dysfunction. Exptl Gerontol, 39:1705-1712
Sinha-Hikim I, Taylor WE, **Gonzalez-Cadavid NF**, Zheng W, Bhasin S (2004) Androgen receptor in human skeletal muscle and cultured muscle satellite cells: Up-regulation by androgen treatment. J Clin Endocr Metabol 89:5245-5255.
Davila H, Magee TR, Rajfer J, **Gonzalez-Cadavid NF** (2005). Peyronie's disease is associated with an increase of plasminogen activator inhibitor-1 (PAI-1) at the RNA and protein levels. Urology. 65:645-648
Hikim AP, Vera Y, Vernet D, Castanares M, Diaz-Romero M, Ferrini M, Swerdloff RS, **Gonzalez-Cadavid NF**, Wang C (2005) Involvement of nitric oxide-mediated intrinsic pathway signaling in age-related increase in germ cell apoptosis in male brown-norway rats. J Gerontol A Biol Sci Med Sci 60:702-708.
Artaza JN, Bhasin S, Magee TR, Reisz-Porszasz S, Shen R, Groome NP, Fareez MM, **Gonzalez-Cadavid NF** (2005) Myostatin inhibits myogenesis and promotes adipogenesis in C3H 10T(1/2) mesenchymal multipotent cells. Endocrinology 146:3547-3557.
Vernet D, Qian A, Nolazco G, Cantini L, Magee TR, Ferrini MG, Rajfer J, **Gonzalez-Cadavid NF** (2005) Evidence that osteogenic progenitor cells in the human tunica albuginea may originate from stem cells. Implications for Peyronie's disease. Biol Reprod, 73:1199-1210.
Jasuja R, Ramaraj P, Mac RP, Singh AB, Storer TW, Artaza J, Miller A, Singh R, Taylor WE, Lee ML, Davidson T, Sinha-Hikim I, **Gonzalez-Cadavid N**, Bhasin S (2005) Delta-4-androstene-3,17-dione binds androgen receptor, promotes myogenesis in vitro, and increases serum testosterone levels, fat-free mass, and muscle strength in hypogonadal men. J Clin Endocrinol Metab. 90:855-863.
Gonzalez-Cadavid NF, Rajfer J (2005) The pleiotropic effects of inducible nitric oxide synthase on the physiology and pathology of penile erection. Curr Pharm Des 11:4041-4046
Gonzalez-Cadavid NF**, Rajfer J (2005) Mechanisms of disease: new insights into the cellular and molecular pathology of Peyronie's disease. Nature Clin Pract Urol, 2:291-298.**
Saenz de Tejada I, Angulo J, Cellek S, **Gonzalez-Cadavid N**, Heaton J, Pickard R, Simonsen U (2005) Physiology of erectile function. J Sex Med 1:254-265.
Saenz de Tejada I, Angulo J, Cellek S, **Gonzalez-Cadavid N**, Heaton J, Pickard R, Simonsen U (2005) Pathophysiology of erectile dysfunction. J Sex Med 2:26-39.
Ferrini MG, Nolazco G, Vernet D, **Gonzalez-Cadavid NF***, Berman J* (*: equal contributors) (2006) Increased vaginal oxidative stress, apoptosis, and inducible nitric oxide synthase in a diabetic rat model: implications for vaginal fibrosis.

Fertil Steril, 86:1152-1163.
Ferrini MG, Kovanecz I, Nolazco G, Rajfer J, **Gonzalez-Cadavid NF** (2006) Effects of long-term treatment with vardenafil on the development of the fibrotic plaque in a rat model of Peyronie's disease BJU Int, 97:625-633
Singh R, Artaza JN, Taylor WE, Braga M, Yuan X, **Gonzalez-Cadavid NF**, Bhasin S (2006) Testosterone inhibits adipogenic differentiation in 3T3-L1 cells: nuclear translocation of androgen receptor complex with beta-catenin and T-cell factor 4 may bypass canonical Wnt signaling to down-regulate adipogenic transcription factors. Endocrinology. 2006 Jan;147(1):141-54.
Vernet D, Magee TR, Qian A, Rajfer J, **Gonzalez-Cadavid NF** (2006) Long-term continuous incubation with high doses of tadalafil does not up-regulate the levels of phosphodiesterase 5 (pde5) in cultures of human penile smooth muscle cells. J Sex Med 3:84-94; discussion 94-95
Magee TR, Artaza JN, Ferrini MG, Zuniga FI, Cantini L, Reisz-Porszasz S, Rajfer J, **Gonzalez-Cadavid NF** (2006). Myostatin shRNA gene therapy increases muscle mass. J Gene Med 8:1171-81
Kovanecz I, Ferrini MG, Vernet D, Nolazco G, Rajfer J, **Gonzalez-Cadavid NF** (2006). Pioglitazone prevents corporal veno-occlusive dysfunction (CVOD) in a rat model of type 2 diabetes melittus. BJU Int, 98:116-124
Ferrini MG, Davila H, Kovanecz I, Sanchez S, **Gonzalez-Cadavid NF*** Rajfer J* (*: equal contributors) (2006**)** Long-term continuous treatment with vardenafil prevents fibrosis and preserves smooth muscle content in the rat corpora cavernosa after bilateral cavernosal nerve transection. Urology, 2006 Aug;68(2):429-35.
Ferrini MG, Kovanecz I, Sanchez S, Vernet D, Davila HH, Rajfer J, **Gonzalez-Cadavid NF** (2007) Long-term continuous treatment with sildenafil ameliorates aging-related erectile dysfunction and the underlying corporal fibrosis. Biol Reprod, 76:915-923
Artaza JN, Reisz-Porszasz S, Dow JS, Kloner RA, Tsao J, Bhasin S, **Gonzalez-Cadavid NF** (2007) Alterations in myostatin expression are associated with changes in cardiac left ventricular mass but not ejection fraction in the mouse. J Endocrinol, 194(1):63-76.
Gonzalez-Cadavid NF, Rajfer J (2007) Experimental models for the study of the cellular and molecular pathophysiology of Peyronie's disease. In: Current Clinical Urology: Peyronie's Disease, A Guide to Clinical Management, ed by L.A. Levine, Humana Press, Totowa, NJ, p 19-39
Magee TR, Kovanecz I, Davila HH, Ferrini MG, Cantini L, Vernet D, Zuniga FI, Rajfer J, **Gonzalez-Cadavid NF** (2007) Antisense and short hairpin RNA (shRNA) constructs targeting PIN (protein inhibitor of NOS) ameliorate aging-related erectile dysfunction in the rat. J Sex Medic, 4(3):633-43.
Kovanecz I, Ferrini MG, Davila HH, Rajfer J, **Gonzalez-Cadavid NF** (2007) Pioglitazone ameliorates penile corpora veno-occlusive dysfunction (CVOD) in the aged rat. BJU Int. 2007 Oct; 100(4):867-74.
Kovanecz I, Rambhatla A, Ferrini MG, Vernet D, Sanchez S, Rajfer J, **Gonzalez-Cadavid NF** (2007) Long term sildenafil treatment ameliorates corporal veno-occlusive dysfunction (CVOD) induced by cavernosal nerve resection in rats. Int J Impot Res, 2007, 100(4):867-74.
Kovanecz I, Rambhatla A, Ferrini MG, Vernet D, Sanchez S, Rajfer J, **Gonzalez-Cadavid** NF (2008) Chronic daily tadalafil prevents the corporal fibrosis and veno-occlusive dysfunction (CVOD) that occurs following cavernosal nerve resection in the rat. BJU Int 101(2):203-10.
Rambhatla A, Kovanecz I, Ferrini M, **Gonzalez-Cadavid NF**, Rajfer J (2008) Rationale for phosphodiesterase 5 inhibitor use post-radical prostatectomy: experimental and clinical review. Int J Impot Res. 20(1):30-4.
Nolazco G, Kovanecz I, Vernet D, Ferrini M, Gelfand B, Tsao J, Magee T, Rajfer J, **Gonzalez-Cadavid NF** (2008) Effect of muscle derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. BJU Int, in press
Artaza JN, Singh R, Ferrini MG, Braga M, Tsao J, **Gonzalez-Cadavid NF** (2008) Myostatin promotes a fibrotic phenotypic switch in multipotent C3h 10T1/2 cells without affecting their differentiation into myofibroblasts. J Endocrinol 196:235-49
Cantini LP, Ferrini MG, Vernet D, Magee TR, Quian A, Gelfand RA, Rajfer J, **Gonzalez-Cadavid NF** (2008) Pro-fibrotic role of myostatin in Peyronie's disease. J Sex Med, in press

D. Ongoing Research Support.

1. G12RR030262 (PI: Kelly, Baker; NGC: Core Director) 10/01/00-09/30/05 Resubmitted for 04/01/08-03/31/13
NIH: RCMI Infrastructure Development Grant Drew bridge funding 10/01/05-ongoing
DNA Repository and Molecular Medicine Core. Renewal as "Molecular Medicine and Stem Cells Core".
This is an institutional support that will cease once the RCMI grant is funded

2. RO1 DK53069-07 (PI: Gonzalez-Cadavid), 05/01/03-04/30/08 NIH/NIDDK

Erectile Dysfunction and Nitric Oxide Synthase in Aging
The goal is to apply novel procedures of gene and stem cell therapy for the treatment of aging-related erectile dysfunction, based on the modulation of the nitric oxide/cGMP pathway in the corpora cavernosa in a rat model of reproductive aging, and whether this restores nitrergic neurotransmission and/or corporal smooth muscle

3. 11881-01R (PI: Gonzalez-Cadavid) 08/01/05-07/31/08 American Diabetes Association
Erectile dysfunction and vascular fibrosis in diabetes
The goal is to study whether the pharmacological modulation of the nitric oxide/cGMP pathway in the corpora cavernosa and the arterial wall prevents fibrosis and loss of compliance in an animal model of type 2 diabetes

4. 512190-01 (PI: Gonzalez-Cadavid) 04/01/07-03/31/08 LABioMed Stem cell seed grant
Characterization of stem cells from human skeletal muscle for the therapy of congestive heart failure
The goal is to determine whether skeletal myoblast preparations from patients with congestive heart failure in an ongoing clinical trial contain stem cells and whether they can undergo cardiomyocyte differentiation

5. PR064756 (PI: Gonzalez-Cadavid) 03/01/07-02/28/10 Department of Defense
Pharmacological prevention and reversion of erectile dysfunction after radical prostatectomy, by modulation of nitric oxide/cGMP pathways
The goal is to determine whether long-term treatment with PDE5 inhibitors and nitric oxide donors can prevent corporal veno-occlusive dysfunction in a rat model of erectile dysfunction after radical prostatectomy, and whether this is due to an improvement in the underlying penile corporal fibrosis and loss of smooth muscle

6. PC061300 (PI: Gonzalez-Cadavid) 03/31/07-02/28/11 Department of Defense
Modulation of stem cell differentiation and myostatin as an approach to counteract fibrosis in dystrophic muscle regeneration after injury.
The goal is to determine whether skeletal muscle derived stem cells (MDSC) can ameliorate skeletal muscle atrophy and fibrosis in a mouse model of Duchenne's muscular dystrophy, and this is stimulated by ex vivo gene transfer of myostatin shRNA to stem cells, and/or treatment with agents that inhibit myostatin activity

7. (Gonzalez-Cadavid) 04/01/07- 03/31/08 Takeda North America, Inc
Antifibrotic and Renoprotective Effects of Pioglitazone on Type 2 Diabetes Related Tubulointerstitial Fibrosis
The goal is to determine whether long-term treatment with pioglitazone reduces vascular and renal fibrosis in an animal model of type 2 diabetes

8. Pilot grant (Bathia/Ho/Gonzalez-Cadavid) 04/01/07-03/31/08 Harbor/UCLA Division of Urodynamics
Reversion of levator ani atrophy by muscle derived stem cells in a rat model of stress urinary incontinence
The goal is to determine whether MDSC can replace defective myofibers, and reduce fibrotic degeneration, in the levator ani of a rat model for female stress urinary incontinence (SUI), and correct SUI

9. NIH R21DK070003-01A1 (Gonzalez-Cadavid) 09/01/07-08/31/09 NIH NIDDK
Cell-selective expression of fibrotic gene pathways
The goal is to compare the patterns of gene expression related to fibrotic phenotypes in smooth muscle and fibroblasts in the corpora cavernosa in rat models of reproductive aging and Peyronie's disease, and the relationship between stem cells, smooth muscle cells, and fibroblasts, in myofibroblast generation in fibrosis.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Monica G. Ferrini	Assistant Professor
eRA COMMONS USER NAME (credential, e.g., agency login) MFerrini306	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Buenos Aires, Argentina School Pharmacy and Biochemistry	M.Sc.	1986	Biochemistry
University of Buenos Aires, Argentina School Pharmacy and Biochemistry	Ph.D.	1995	Physiology

A. POSITIONS

1992-2001 Assistant Professor. University of Buenos Aires, Argentina
1994-2001 Member of the National Research Council of Argentina (CONICET). Current rank: Adjunct Investigator.
1999-2007 Research Associate, LA BioMed at Harbor UCLA, Los Angeles, CA
2004-on Assistant Research Professor, David Geffen School Med at UCLA, Department of Urology, Los Angeles, CA
2004-on Assistant Professor, Department Biomedical Sciences, College Health and Science, Charles R. Drew University, Los Angeles, CA.
2007-on Assistant Professor, Department of Internal Medicine, College of Medicine, Charles Drew University.

HONORS

1993. "Dr Juan Izquierdo Award" Argentine Society Experimental Pharmacology (SAFE)
1991-1995 "Scientific and Technological Awards University of Buenos Aires"
1996. 3rd place in the list of Young Oustanding Researcher, University of Buenos Aires. Argentina.
1997. "Mr Julio Lutfi Award" for the best young endocrinology researcher. Obtained diploma for second place . Argentine Society of Endocrinology and Metabolism.
2000. "AUA: Second place best poster category "Impotence"
2004. Travel Grant Award. Endocrine Society.

B. PUBLICATIONS selected out of total of 52 papers

1. **Ferrini M**, Magariños A.M, De Nicola AF (1990) Estrogens down-regulate type I but not type II adrenal corticoids receptors in rat anterior pituitary. J Steroid Biochem Mol Biol 35: 671-677.
2. **Ferrini M**, De Nicola AF. (1991) Estrogens up-regulate Type I and Type II glucocorticoid receptors in brain regions from ovariectomized rats. Life Sci 48 (26) 2593-2601.
3. **Ferrini M.**, González S, De Nicola AF. (1993) Estradiol increases glucocorticoid binding and glucocorticoid induction of ornithine decarboxilase in the rat spinal cord. Life Sci, 52: 677-685.
4. **Ferrini M.**, González S, Antakly T, De Nicola AF. (1993) Immunocytochemical localization of glucocorticoid receptors in the spinal cord: Effects of adrenalectomy, glucocorticoid treatment and spinal cord transection. Cell Mol Neurobiol, 13/4: 387-397.
5. **Ferrini, M.**, Lima A, De Nicola AF. (1995) Estradiol abolishes down regulation of glucocorticoid receptors in brain. Life Sci 57: 2403:2412.
6. **Ferrini, M.**, Grillo, C, Piroli, G, De Kloet, ER, De Nicola, AF. (1997) Sex difference in glucocorticoid regulation of vasopressin mRNA in the paraventricular hypothalamic nucleus. Cell Mol Neurobiol, 17: 671-686.
7. De Nicola, AF, **Ferrini, M.**, González, S, González Deniselle, MC, Grillo, C, Piroli, G, Saravia, S, De

Kloet, ER. (1998) Regulation of gene expression by corticoid hormones in the brain and spinal cord. J. Steroid Biochem Mol Biol, 65: 253-272.
8. **Ferrini, M**., Piroli G, Grillo,C, González-Deniselle,MC, Lima, A, Roig, P, De Nicola, AF. (1998) Effect of estrogen on the immunoreactivity of choline acetyl transferase (CHAT) and mRNA of GAP-43 in aged rats Act Physiol Latinoam 78 : 48 abt 23
9. **Ferrini, M.,** Piroli, G, Frontera M, Falbo, A, Lima, AE. (1999) Estrogen normalize the response to stress and increase glucocorticoid receptors immunoreactivity in aging rats. Neuroendocrinology 69 129-137
10. Gonzalez Deniselle, M.C., Lavista-Llanos, S., **Ferrini, M.,** Lima A.E., Roldan A.G., De Nicola A.F.: (1999) In vitro differences between astrocytes of control and wobbler mice spinal cord. Neurochem.Res**,** 24: 1531-1541
11. Bisagno, V., **Ferrini, M**., Rios H., Ziehr, L M and Wikinski S. I: (2000) Chronic corticosterone impairs inhibitory avoidance in rats: possible link with hippocampal CA3 dendritic atrophy. Pharmacol Biochem Behav. 66(2):235-40.
12. **Ferrini M.**, Magee, TR, Vernet D, Rajfer J, Gonzalez-Cadavid, NF (2001) Aging-related expression of inducible nitric oxide synthase and markers of tissue damage in the rat penis. Biol Reprod 64: 974-982.
13. **Ferrini M.**, Wang C, Swerdloff R, Vernet D, Sinha-Hikim A, Gonzalez-Cadavid NF. (2001) Aging related expression of inducible nitric oxide synthase (iNOS) and cytotoxicity markers in the rat hypothalamic regions associated with male reproductive dysfunctions. Neuroendocrinology 74: 1-11.
14. **Ferrini M.**, Vernet D, Magee TR, Shahed A, Qian A, Rajfer J, Gonzalez-Cadavid NF. (2002) Anfibrotic role of inducible nitric oxyde synthase. Nitric Oxide: Biol Chem 6:283-294
15. Magee TR, **Ferrini M**., Garban H, Vernet D, Mitani K, Rajfer J, Gonzalez-Cadavid NF (2002) Gene therapy of erectile dysfunction in the rat with penile neuronal nitric oxide synthase (PnNOS) cDNA. Biol Reprod. 67:20-28
16. **Ferrini M**., Bisagno V, Piroli G, Grillo, C, Gonzalez-Deniselle, MC, De Nicola AF. (2002) Effects of estrogens on choline-acetyltransferase immunoreactivity and GAP-43 mRNA in the forebrain of young and aging male rats. Cell Mol Neurobiol, 22:289-301.
17. Gonzalez-Cadavid NF, Magee TR**, Ferrini M**., Qian A, Vernet D, Rajfer J (2002) Gene expression in Peyronie's disease. In: "Update of Peyronie's disease", ed. by Nera A, Hellstrom W. Intn J Impot Res, 14(5): 361-374.
18. Vernet, D, **Ferrini MG,** Valente EG, Magee TR, Bou Gahrios G, Rajfer, J, Gonzalez-Cadavid, N.F. (2002) Effect of nitric oxide on the differentiation of fibroblasts into myofiobroblasts in the Peyronie's fibrotic plaque and in its rat model. Nitric oxide, Biol Chem 7:262-276.
19. **Ferrini M**, Magee TR, Vernet D, Rajfer J, Gonzalez-Cadavid NF (2002) Aging-related expression of inducible nitric oxide synthase and markers of tissue damage in the rat penis. Int J Impot Res 14:550 Editorial commentary
20. Magee, TR, **Ferrini, MG.**, Davila, H, Zeller, CB, Vernet, D, Sun, J, Lalani, R, Burnett, AL, Rajfer, J, Gonzalez-Cadavid, NF. (2003) The protein inhibitor of NOS (PIN) and the NMDAR receptor are expressed in the rat and mouse penile nerves and co-localize with penile neuronal nitric oxide synthase. Biol Reprod 68:478-88.
21. **Ferrini, MG.**, Magee, TR, Vernet, D, Rajfer, J, Gonzalez-Cadavid, NF. (2003) Penile neuronal nitric oxide synthase (PnNOS) and its regulatory proteins are present in hypothalamic regions involved in the control of penile erection. J Comp Neurol 458(1): 46-61.
22. Davila HH, **Ferrini MG**., Rajfer J, Gonzalez-Cadavid NF (2003) Fibrin as an inducer of fibrosis in the tunica albuginea of the rat: a new animal model of Peyronie's disease. Brit J Urol Int 91:830-838.
23. Valente EG, **Ferrini MG**, Vernet D, Qian A, Rajfer J, Gonzalez-Cadavid NF (2003) L-arginine and PDE inhibitors counteract fibrosis in the Peyronie's fibrotic plaque and related fibroblast cultures. Nitric Oxide, 9(4): 229-244.
24. **Ferrini M**, Davila HH, Valente EG, Gonzalez-Cadavid NF, Rajfer J. (2004) Aging-related induction of inducible nitric oxide synthase is vasculo-protective to the arterial media. Cardiovascular Res., 61(4):796-805.
25. Hikim AP, Vera Y, Vernet D, Castanares M, Diaz-Romero M, **Ferrini M**,Swerdloff RS, Gonzalez-Cadavid NF, Wang C (2005). Involvement of nitric oxide-mediated intrinsic pathway signaling in age-

related increase in germ cell apoptosis in male Brown-Norway rats. J Gerontol A Biol Sci Med Sci. 60(6):702-708.
26. **Ferrini MG**, Kovanecz I, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. (2006) Effects of long-term vardenafil treatment on the development of fibrotic plaques in a rat model of Peyronie's disease.BJU Int. 97(3):625-33.
27. **Ferrini MG**, Nolazco G, Vernet D, Gonzalez-Cadavid NF, Berman J. Increased vaginal oxidative stress, apoptosis, and inducible nitric oxide synthase in a diabetic rat model: implications for vaginal fibrosis. Fertil Steril. 2006 Oct;86 Suppl 4:1152-63.
28. De Nicola AF, Saravia FE, Beauquis J, Pietranera L, **Ferrini MG.** Estrogens and neuro-endocrine hypothalamic-pituitary-adrenal axis function.Front Horm Res. 2006;35:157-68.
29. **Ferrini MG,** Davila HH, Kovanecz I, Sanchez SP, Gonzalez-Cadavid NF, Rajfer J. Vardenafil prevents fibrosis and loss of corporal smooth muscle that occurs after bilateral cavernosal nerve resection in the rat.Urology. 2006 Aug;68(2):429-35
30. Kovanecz I, **Ferrini MG**, Vernet D, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. Pioglitazone prevents corporal veno-occlusive dysfunction in a rat model of type 2 diabetes mellitus. BJU Int. 2006 Jul; 98(1):116-24.
31. Magee TR, Artaza JN, Ferrini MG, Vernet D, Zuniga FI, Cantini L, Reisz-Porszasz S, Rajfer J, Gonzalez-Cadavid NF. Myostatin short interfering hairpin RNA gene transfer increases skeletal muscle mass. J Gene Med. 2006 Sep;8(9):1171-81.
32. Paez Espinosa V, **Ferrini M**, Shen X, Lutfy K, Nillni EA, Friedman TC. Cellular co-localization and co-regulation between hypothalamic pro-TRH and prohormone convertases in hypothyroidism. Am J Physiol Endocrinol Metab. 2007 292(1):E175-86
33. **Ferrini MG,** Kovanecz I, Sanchez S, Vernet D, Davila HH, Rajfer J,Gonzalez-Cadavid NF. Long-Term Continuous Treatment with Sildenafil Ameliorates Aging-Related Erectile Dysfunction and the Underlying Corporal Fibrosis in the Rat. Biol Reprod. 2007, 73: 915-923
34. Magee TR, Kovanecz I, Davila HH, **Ferrini MG**, Cantini L, Vernet D, Zuniga FI, Rajfer J, Gonzalez-Cadavid NF. Antisense and short hairpin RNA (shRNA) constructs targeting PIN (Protein Inhibitor of NOS) ameliorate aging-related erectile dysfunction in the rat.J Sex Med. 2007 May;4(3):633-43.
35. Khorram O, Momeni M, **Ferrini M,** Desai M, Ross MG.In utero undernutrition in rats induces increased vascular smooth muscle content in the offspring. Am J Obstet Gynecol. 2007 May;196(5):486.e1-8.
36. Kovanecz I, **Ferrini MG**, Vernet D, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. Ageing-related corpora veno-occlusive dysfunction in the rat is ameliorated by pioglitazone. BJU Int. 2007 Oct;100(4):867-74.
37. Rambhatla A, Kovanecz I, **Ferrini M**, Gonzalez-Cadavid NF, Rajfer J. Rationale for phosphodiesterase 5 inhibitor use post-radical prostatectomy: experimental and clinical review. Int J Impot Res. 2007 Aug 2; [Epub ahead of print]
38. Kovanecz I, Rambhatla A, **Ferrini M**, Vernet D, Sanchez S, Rajfer J, Gonzalez-Cadavid N. Long-term continuous sildenafil treatment ameliorates corporal veno-occlusion dysfunction (CVOD) induced by cavernosal nerve resection in rats. Int J Impot Res. 2007 Sep 20; [Epub ahead of print]
39. Kovanecz I, Rambhatla A, **Ferrini MG**, Vernet D, Sanchez S, Rajfer J, Gonzalez-Cadavid N. Chronic daily tadalafil prevents the corporal fibrosis and veno-occlusive dysfunction that occurs after cavernosal nerve resection. BJU Int. 2007 Sep 20; [Epub ahead of print]
40. J.N. Artaza, R. Singh, **M.G. Ferrini,** M. Braga J. Tsao Myostatin promotes a fibrotic phenotypic switch in multipotent C3H 10T1/2 cells without affecting their differentiation into myofibroblasts. and N. Gonzalez-Cadavid. Journal of Endocrinology, 2008 Feb; 196 (2):235-249.
41. Cantini LP, **Ferrini MG**, Vernet D, Magee TR, Qian A., Gelfand RA, Rajfer J., Gonzalez-Cadavid NF: Pro-fibrotic role of myostatin in Peyronie's Disease J. Sex. Med. 2008 in press.
42. Nolazco G, Kovanecz I, Vernet D, **Ferrini MG**, Gelfand B, Tsao J, Magee T, Rajfer J, Gonzalez-Cadavid NF (2007) Effect of muscle derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. BJU Int, in press

C. OTHER SUPPORT

Active

Active/Pending:	Active
Project Number (Principal Investigator):	1P20-MD-000545-01 (Sayed, Gary, Dean of COSH, Drew Univ.)
Source:	NIH
Title of Project (*and/or Subproject*):	Charles R Drew University College of Allied Health Undergraduate Program
Dates of Approved/Proposed Project:	10/1/05 - 9/30/08

Annual Direct Costs / Percent Effort:

The College of Allied Health plans to enhance its current academic health and life science programs by creating an undergraduate program in Biomedical Science. To Achieve its goals, four areas will be implemented: 1. Faculty Development; 2. Physical Plant Development 3. Career Development. This would lead toward the eventual development of a strong educational biomedical research Bachelor degree program, that would serve as an educational pipeline to the College of Medicine at Drew or UCLA

Active/Pending:	Active
Project Number (Principal Investigator):	Log #PR064756, PR#W91ZSQ-6289-N634 Pi: Gonzalez-Cadavid.
Source:	Department of Defense
Title of Project (*and/or Subproject*):	Modulation of stem cell differentiation and myostatin activity as an approach to counteract fibrosis in muscle dystrophy and regeneration after injury
Dates of Approved/Proposed Project:	03/01/07-2/28/11
Annual Direct Costs:	197,247
Person months (Calendar months):	4.8 month

Goals: The goal is to investigate in the mdx mouse a novel therapeutic approach for **DMD** based on the inhibition of myostatin **(Mst)** expression and/or activity, for the alleviation of fibrotic and fatty degeneration of the muscle, that would also facilitate the differentiation of transplanted dystrophin+ **(D+)** muscle-derived stem cells **(MDSC),** in order to ameliorate disease progression

Overlap: None.

Active/Pending:	Pending Award
Project Number (Principal Investigator):	1SC1GM038706-01. PI: Ferrini, Monica G
Source:	NIH
Title of Project (*and/or Subproject*):	Nitric oxide/cGMP modulation of corporal fibrosis caused by neuropraxia
Dates of Approved/Proposed Project:	04/01/08-3/31/13
Annual Direct Costs:	200,000
Person months (Calendar months):	4.8 month

Goals: To define whether PDE5 inhibitors alone or in combination with other drugs that also up-regulate the NO/cGMP pathway, correct not only the underlying histopathology of the corpora but also preserve the normal physiology of the tissue. In addition, to clarify the mechanism of these effects by determining a) to what extent different damaged tissues are affected by these agents; b) what role nitric oxide (NO) and cGMP have on correcting oxidative stress as a factor inducing corporal tissue damage after surgery; and c) what are the downstream targets of NO and cGMP when there is amelioration of corporal tissue damage.

Overlap: None.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
KOVANECZ, ISTVAN	Assistant Professor (appointment in progress)
eRA COMMONS USER NAME (credential, e.g., agency login) IKOVANECZ0308	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Szeged (former Jozsef A. University of Art and Sciences), Szeged, Hungary	M.Sc.	1985	Biochemistry
Budapest University of Technology, Institute of Continuing Engineering Education, Budapest, Hungary	CNRT	1987	Nuclear technology
University of Szeged (former Jozsef A. University of Art and Sciences), Szeged, Hungary	Ph.D	1994	Comparative Physiology
Biological Research Center of The Hungarian Academy of Sciences, Szeged, Hungary		1999-2000	Genomics, IT, Bioinformatics

A. Positions and Honors

1985-1987 Research Fellow, Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary
1987-1991 Research Scientist, Blood Transfusion Center, Szent-Gyorgyi Albert Medical University, Szeged, Hungary
1991-1992 Volunteer Researcher, Department of Neurology, Mount Sinai Medical Center, CUNY, New York, NY, USA
1993-1999 Senior Research Scientist, Head of the Vivarium, Department of Pharmacology and Pharmacotherapy, Szent-Gyorgyi Albert Medical University, Szeged, Hungary
1999-2001 Biologist Chief Counselor, Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary
2000-2001 Member of the Computer Software Council of the Hungarian Academy of Sciences
2004–on Research Associate, Urology Research Laboratory, Department of Surgery, Los Angeles Biomedical Research Institute at Harbor-UCLA, Torrance, CA, USA
2008-on Assistant Professor (in progress), Department of Urology, UCLA David Geffen School of Medicine, Los Angeles, CA, USA

B. Professional membership

1982–96 John von Neumann Society of Computer Sciences
1993–01 Hungarian Genetical Society
1994–99 Hungarian Society of Cardiology
1994–99 Hungarian Society for Experimental and Clinical Pharmacology
1994–99 International Society for Heart Research (European Section)
2000–01 Computer Software Council of the Hungarian National Academy of Sciences
2006–on National Geographic Society
2007–on Sexual Medicine Society of North America
2008–on American Urological Association

C. Selected publications

Original research and theoretical treatises

1. Bodis-Wollner I, Antal A, **Kovanecz I**. Low-dose scopolamine and acetyl-levo-carnitine dissociate primary from cognitive visual processing in the trained monkey. *Invest Ophth Vis Sci* 1993; 34(4): 1174.
2. Antal A., **Kovanecz I,** Bodis-Wollner I. Visual discrimination and P300 are affected paralell by cholinergic agents in the behaving monkey. *Physiol Behav.* 1994; 56(1) 161-66.
3. Tagliati M., Bodis-Wollner I., **Kovanecz I,** Stanzione P. Spatial frequency tuning in the monkey retina depends on D2 receptor-linked action of dopamine. *Vision Research* 1994; 34(16):22051-57.
4. **Kovanecz I**, Csajbok E., Petri I.B. In vitro steroid sensitivity in chronic uremic and kidney transplant patients: HLA - associated susceptibility to steroid treatment. *Nephrology Dialysis Transplantation* 1994; 9(10): 1474-76.
5. **Kovanecz I**, Petri I.B., Kaiser G. HLA associated lymphocyte panel reactive (cytotoxic) antibody production in dialyzed chronic uremic patients. *Acta Microbiologica Hungarica* 1995; 42(1): 81-84.
6. **Kovanecz I,** Papp JG., Szekeres L. Increased cardiac workload by adrenoreceptor agonists for the estimation of potential antiischemic activity in a conscious rabbit model. *J Pharmacol Toxicol Methods* 1997; 37(3): 149-59.
7. Szekeres L, **Kovanecz I**, Papp JG. Delayed cardiac adaptation to stress moderates response to beta-adrenoceptor agonists. *J Mol Cell Cardiol* 1997; 29(5): A134.
8. **Kovanecz I**, Ábrahám A, Makay G, Lukács E, Szekeres L, Papp JGy. Delayed cardiac adaptation to ischaemic stress - limitation of infarct size in a rabbit model of ischaemia-reperfusion by a single dose of iloprost. 1997 . *J Mol Cell Cardiol* 1997; 29(5): A89.
9. Takase H, **Kovanecz I**, Mori T. et al. Acute and anti-ischemic actions of pranipidine in three animal models. *Asia Pacific Journal of Pharmacology* 2003; 16(1): 29-37.
10. Davila HH, Miranda-Sousa AJ, **Kovanecz I**, et al. Effect of bilateral cavernosal nerve resection on the histological alteration in the penile vascular system. *J Urol* 2005; 173(4S): 288.
11. Ferrini MG, **Kovanecz I**, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. Effects of long-term vardenafil treatment on the development of fibrotic plaques in a rat model of Peyronie's disease. *BJU Int*. 2006 Mar; 97(3):625-33.
12. **Kovanecz I**, Ferrini MG, Vernet D, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. Pioglitazone prevents corporal veno-occlusive dysfunction (CVOD) in a rat model of type 2 diabetes mellitus. *BJU Int*. 2006; 98:116-24
13. Ferrini MG, Davila HH, **Kovanecz I**, Sanchez SP, Gonzalez-Cadavid NF, Rajfer J. Vardenafil prevents fibrosis and loss of corporal smooth muscle that occurs after bilateral cavernosal nerve resection in the rat. *Urology* 2006; 68:429-35
14. Ferrini MG, **Kovanecz I**, Sanchez S, Vernet D, Davila HH, Rajfer JA, Gonzalez-Cadavid NF. Long-term continuous treatment with sildenafil ameliorates aging-related erectile dysfunction and the underlying corporal fibrosis in the rat. *Biol Reprod.* 2007; 76(5):915-23.
15. Magee TR, **Kovanecz I**, Davila HH, Ferrini MG, Cantini L, Vernet D, Zuniga FI, Rajfer J, Gonzalez-Cadavid NF. Antisense and short hairpin RNA (shRNA) constructs targeting PIN (Protein Iinhibitor of NOS) ameliorate aging-related erectile dysfunction in the rat. *J Sex Med* 2007; 4(3):633-43.
16. **Kovanecz I**, Ferrini MG, Vernet D, Nolazco G, Rajfer J, Gonzalez-Cadavid NF. Aging-related copora veno-occlusive dysfunction in the rat is ameliorated by pioglitazone. *BJU Int* 2007; 100(4):867-74.
17. **Kovanecz I**, Rambhatla A, Ferrini MG, Rajfer J, Gonzalez-Cadavid NF. Long term sildenafil treatment ameliorates corpora veno-occlusive dysfunction (CVOD) induced by cavernosal nerve resection in rats. *Int J Impot Res* 2007 Sep 20; [Epub ahead of print]
18. **Kovanecz I**, Rambhatla A, Ferrini MG, Rajfer J, Gonzalez-Cadavid NF. Chronic daily tadalafil prevents the corporal fibrosis and veno-occlusive dysfunction that occurs after cavernosal nerve resection. *BJU Int.* 2008; 101(2):203-10.
19. Nolazco D, **Kovanecz I**, Vernet D, Ferrini MG, Gelfand B, Tsao J, Mage T, Rajfer J, Gonzalez-Cadavid NF. Effect of muscle derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. *BJU Int* 2008 Feb 21; [Epub ahead of print]

Non-experimental articles

1. Rambhatla A, **Kovanecz I,** Ferrini M, Gonzalez-Cadavid NF, Rajfer J. Rationale For PDE5 Inhibitor Use Post Prostatectomy. *Int J Impot Res* 2008; 20(1):30-34. [Epub 2007 Aug 2.]

D. Ongoing Research Support

None

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Vernet, Agueda Dolores	Research Associate
eRA COMMONS USER NAME (credential, e.g., agency login) avernet0507	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Central Univ of Venezuela, Caracas, Venezuela	Biologist	1976	Cellular Biology
Venezuelan Inst Scientific Res, Venezuela	M.Sc.	1983	Immunology
Central Univ of Venezuela, Caracas, Venezuela	Ph.D.	1993	Cellular Biology

A. Postitions and Honors

1976-83 Biologist, Institute of Legal Medicine, Caracas, Venezuela
1980-81 Visiting Scientist, University of Compiègne, Compiègne, France
1983-84 Biologist, Amazonic Center of Research of Tropical Diseases, Caracas, Venezuelas Instructor
Professor, Dept Biochem, Fac Medicine, Central Univ Venezuela, Caracas, Venezuela.
1991 Research Associate, Dept Surgery, Harbor-UCLA Medical Center, Torrance, California
1992 Assoc Professor, Dept Pathophysiol, Fac.Medicine, Central Univ Venezuela, Caracas, Venezuela.
1993-on Research Associate, Dept Surgery, LABiomed at Harbor-UCLA Med Center, Torrance, CA
2004-on Research Associate (part time), NIH RCMI Program, Charles Drew University, Los Angeles, CA

B. Publications

1. San Blas G, **D Vernet** (1975) Induction of the synthesis of the cell wall α-1,3-glucan in the yeast-like form of Paracoccidioides brasiliensis strain IVIC Pb9 by fetal calf serum. Infect & Immun 15:897-902.
2. Gonzalez-Cadavid NF, **D Vernet,** A Fuentes, JA Rodriguez, RS Swerloff, J Rajfer (1993) Up-regulation of levels of androgen receptor and its mRNA by androgens in smooth-muscle cells from rat penis. Molec Cell Endocrinol 90:219-229
3. Garban H, **D Vernet**, A Freedman, J Rajfer, NF Gonzalez-Cadavid (1995) Effect of aging on nitric oxide-mediated penile erection in rats. Am J Physiol 268: (Heart Circ Physiol) H467-H475
4. Hung A, **D Vernet**, Y Xie, T Rajavashisth, JA Rodriguez, J Rajfer, NF Gonzalez-Cadavid (1995) Expression of the inducible nitric oxide synthase in smooth muscle cells from the rat penile corpora cavernosa. J Androl 16:469-481
5. **Vernet D**, L Cai, H Garban, ML Babbit, F Murray, J Rajfer, NF Gonzalez-Cadavid (1995) Reduction of penile nitric oxide synthase in diabetic BB/WOR^{dp} (Type I) rats with erectile dysfunction. Endocrinology 136:5709-5717.
6. Garban H, D Marquez, T Magee, J Moody, T Rajavashisht, JA Rodriguez, A Hung, **D Vernet**, J Rajfer, NF Gonzalez-Cadavid (1997) Cloning of rat and human inducible nitric oxide synthase. Application for gene therapy of erectile dysfunction. Biol Reproduct 56, 954 -963.
7. Moody J, **Vernet D**, S Laidlaw, J Rajfer, NF Gonzalez-Cadavid (1997) Effects of long-term administration of L-arginine on the rat erectile response. J Urol 158, 942-947

8. **Vernet D**, Bonavera JJ, Swerloff R, Gonzalez-Cadavid NF, Wang C. (1998) Spontaneous expression of inducible nitric oxide synthase (iNOS) in the hypothalamus and other brain regions of aging rats. Endocrinology, 139(7): 3254-3261

9. González-Cadavid NF, Burnett AL, Magee T, Zeller CB, **Vernet D**, Gitter J, Smith N, Rajfer J (2000) Expression of penile neuronal nitric oxide synthase variants in the rat and mouse penile nerves. Biol Reprod, 63:704-714

10. González-Cadavid NF, Ryndin I, **Vernet D**, Magee TR, Rajfer J (2000) Presence of NMDA receptor subunits in the male lower urogenital tract. J Androl 21:566-578

11. Ferrini M, Magee TR, **Vernet D**, Rajfer J, Gonzalez-Cadavid NF (2001) Aging-related expression of inducible nitric oxide synthase (iNOS) and markers of tissue damage in the rat penis. Biol Reprod, 64:974-982

12. Wang C, Sinha Hikim A, Ferrini M, Bonavera JJ, **Vernet D**, Leung A, Lue-YH, Gonzalez-Cadavid NF, Swerdloff RS (2002) Male reproductive aging: using the Brown Norway rat as a model for man. In: "Endocrine facets of aging in the human and experimental animal" Novartis Found Symp 242:82-95

13. Ferrini MG, **Vernet D**, Magee TR, Shahed A, Quian A, Rajfer J, Gonzalez-Cadavid NF (2002) Antifibrotic role of inducible nitric oxide synthase (iNOS). Nitric Oxide 6:1-12

14. Gonzalez-Cadavid NF, Magee TR, Ferrini M, Qian A, **Vernet D**, Rajfer J (2002) Gene expression in Peyronie's disease. Int J Impot Res, 14:361-374**.**

15. Magee TR, Ferrini M, Garban H, **Vernet D**, Mitani K, Rajfer J, Gonzalez-Cadavid NF (2002) Gene therapy of erectile dysfunction in the rat with penile neuronal nitric oxide synthase cDNA. Biol Reprod, 67:1033-1041.

16. **Vernet D**, Ferrini MG, Valente E, Magee TR, Bou-Gharios G, Rajfer J, Gonzalez-Cadavid NF (2002) Effect of nitric oxide on fibroblast differentiation into myofibroblasts in cell cultures from the Peyronie's fibrotic plaque and in its rat model in vivo. Nitric Oxide 7:262-276.

17. Ferrini MG, Magee TR, **Vernet D**, Rajfer J, González-Cadavid NF (2003) Penile neuronal nitric oxide synthase (PnNOS) and its regulatory proteins are present in hypothalamic and spinal cord regions involved in the control of penile erection. J Compar Neurol 458:46-61

18. Magee TR, Ferrini MG, Davila HH, Zeller CB, **Vernet D**, Sun J, Lalani R, Burnett AL, Rajfer J, González-Cadavid NF. (2003) Protein inhibitor of nitric oxide synthase (NOS) and the N-methyl-D-aspartate receptor are expressed in the rat and mouse penile nerves and colocalize with penile neuronal NOS. Biol Reprod 68:478-488.

19. Valente EG, **Vernet D**, Ferrini MG, Qian A, Rajfer J, González-Cadavid NF (2003) L-arginine and (PDE) inhibitors counteract fibrosis in the Peyronie's fibrotic plaque and related fibroblast cultures. Nitric Oxide, 9:229-244

20 Davila HH, Magee TR, **Vernet D**, Rajfer J, González-Cadavid NF. (2004) Gene transfer of inducible nitric oxide synthase cDNA regresses the fibrotic plaque in an animal model of Peyronie's disease. Biol Reprod 71(5).90

21. Sinha Hikim AP, Vera Y, **Vernet D**, Castanares M, Diaz-Romero M, Ferrini M, Swerdloff RS, González-Cadavid NF, Wang C. (2005) Involvement of nitric oxide-mediated intrinsic pathway signaling in age-related increase in germ cell apoptosis in male brown-norway rats. J Gerontol A Biol Sci Med Sci. 60(6):702-8.

22. **Vernet D**, Qian A, Nolazco G, Cantini L, Magee TR, Ferrini MG, Rajfer J, González-Cadavid NF (2005) Evidence that osteogenic progenitor cells in the human tunica albuginea may originate from stem cells. Implications for Peyronie's disease. Biol Reprod, 73:1199-1210

23. **Vernet D**, Magee TR, Qian A, Nolazco G, Rajfer J, González-Cadavid NF. (2006) Phosphodiesterase type 5 is not upregulated by tadalafil in cultures of human penile cells. J Sexual Medicine,3:84-95.

25. Magee TR, Artaza JN, Ferrini MG, **Vernet D**, Zuniga FI, Cantini L, Reisz-Porszasz, Rajfer J, González-Cadavid, NF. (2006) Myostatin short interfering hairpin RNA gene transfer increases muscle mass. J Gene Med, 8(9):1171-81.

26. Kovanecz I, Ferrini MG, **Vernet D**, Nolazco G, Rajfer J, González-Cadavid NF (2006). Pioglitazone prevents corporal veno-occlusive dysfunction in a rat model of type 2 diabetes mellitus. British J Urology, 98(1):116-124

27. Ferrini MG, Nolazco G, **Vernet D**, González-Cadavid NF, Berman J. (2006). Increased vaginal oxidative stress, apoptosis, and inducible nitric oxide synthase in a diabetic rat model: implications for vaginal fibrosis. Fertil Steril, 86 Suppl 4:1152-63; [Epub ahead of print]

28. Ferrini MG, Kovanecz I, Sanchez S, **Vernet D**, Davila HH, Rajfer J, Gonzalez-Cadavid NF. (2007) Long-Term Continuous Treatment with Sildenafil Ameliorates Aging-Related Erectile Dysfunction and the Underlying Corporal Fibrosis in the Rat. Biol Reprod Feb 14; [Epub ahead of print]

29. Magee TR, Kovanecz I, Davila H, Ferrini M, Cantini L, **Vernet D**, Zuniga F, Rajfer J, Gonzalez-Cadavid NF. (2007) Antisense and short hairpin RNA (shRNA) constructs targeting PIN (Protein Inhibitor of NOS) ameliorate aging-related erectile dysfunction in the rat. J Sex Med, 4(3):633-43

30. Kovanecz I, Rambhatla A, Ferrini MG, **Vernet D**, Sanchez S, Rajfer J, Gonzalez-Cadavid NF (2007) Long term sildenafil treatment ameliorates corporal veno-occlusive dysfunction (CVOD) induced by cavernosal nerve resection in rats. Int J Impot Res, 2007, 100(4):867-74.

31. Kovanecz I, Rambhatla A, Ferrini MG, **Vernet D**, Sanchez S, Rajfer J, Gonzalez-Cadavid NF (2008) Chronic daily tadalafil prevents the corporal fibrosis and veno-occlusive dysfunction (CVOD) that occurs following cavernosal nerve resection in the rat. Brit J Urol Int 101(2):203-10.

32. Nolazco G, Kovanecz I, **Vernet D**, Ferrini M, Gelfand B, Tsao J, Magee T, Rajfer J, Gonzalez-Cadavid NF (2008) Effect of muscle derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. British JUrol Int, in press

33. Cantini LP, Ferrini MG, **Vernet D**, Magee TR, Quian A, Gelfand RA, Rajfer J, Gonzalez-Cadavid NF (2008) Pro-fibrotic role of myostatin in Peyronie's disease. J Sex Med, in press

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Gaby Nolazco	Research Associate
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
California State University Fullerton	M.Sc.	2003	Biological Science

A. Positions and Honors.

2004-2007 Research Associate at Dept Surgery/Urology Research Laboratory Los Angeles Biomedical Research Institute at Harbor-UCLA, Torrance CA.
2007- on Research Associate at Charles Drew University School of Medicine, Los Angeles CA.

B. Publications.

1. Nolazco, G.M. and Ramirez, J. (2000) Determination of specific acetylcholine receptor subunit expression in identified neurons found in the marine mollusk, *Aplysia californica*. *Dimensions*, 2: 14-19. (Undergraduate student research publication at CSU Fullerton)

2. Nolazco, G.M. (2003) Identification and developmental expression of alternatively spliced transcripts of the small conductance calcium-activated potassium channel in *Drosophila melanogaster*, Master's Thesis, California State University Fullerton.

3. Vernet D., **Nolazco G.**, Cantini L., Magee TR., Qian A., Rajfer J., Gonzalez-Cadavid NF. (2005) Evidence that osteogenic progenitor cells in the human tunica albuginea may originate from stem cells: implications for peyronie disease. Biol Reprod, 73(6): 1199-210.

4. Vernet D., Magee TR., Qian A., **Nolazco G.,** Rajfer J., Gonzalez-Cadavid NF. (2006) Phosphodiesterase type 5 is not upregulated by Tadalafil in cultures of human penile cells. J Sex Medicine, (3(1):84-95.

5. Ferrini, MG., Kovanecz, I., **Nolazco, G**., Rajfer, J., Gonzalez-Cadavid NF (2006) Effects of long-term vardenafil treatment on the development of fibrotic plaques in a rat model of Peyronie's disease. Br J Urol, 97 (3): 625-33.

6. Kovanecz, I., Ferrini, M.G., Vernet, D., **Nolazco, G**., Rajfer, J., Gonzalez-Cadavid NF (2006) Pioglitazone prevents corporal veno-occlusive dysfunction (CVOD) in a rat model of type 2 diabetes mellitus. Br J Urol , 98(1):116-24.

7. Ferrini, M., **Nolazco, G**., Vernet, D., Gonzalez-Cadavid, N., Berman, J. (2006) Increased vaginal oxidative stress, apoptosis, and inducible nitric oxide synthase, in a diabetic rat model: implications for vaginal fibrosis. J Fertil and Steril, 86 Suppl 4: 1152-63.

8. Kovanecz, I., Ferrini, M.G., Vernet, D., **Nolazco, G**., Rajfer, J., Gonzalez-Cadavid NF (2007) Ageing-related corpora veno-occlusive dysfunction in the rat is ameliorated by pioglitazone. Br J Urol, 100 (4)867-74.

9. Nolazco, G., Kovanecz, I., Vernet, D., Gelfand RA., Tsao J., Ferrini, MG., Magee, T., Rajfer, J., Gonzalez-Cadavid, NF. (2008) Effect of muscle derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. Br J Urol, Epub ahead of print.

Attending Meeting.

1. Nolazco, G.M., Ramirez, J., and Ono, J.K. (1999) Determination of specific acetylcholine receptor subunits expressed in identified *Aplysia* neurons.
Minority Scientist Symposium. Phoenix, Arizona

2. Amer, A., **Nolazco, G.,** R. E. McCaman and J.K. Ono (2000), Comparisons of nicotinic acetylcholine receptor alpha subunits in muscles and nervous systems of mollusks and annelids, *Soc. Neurosci. Abstracts*, *26*: 1167.

3. Nolazco, G.M., Ramirez, J., and Ono, J.K. (2000) Determination of specific acetylcholine receptor subunits expression in identified neurons: LP1 and BR2.
California State University Program for Education and Research in Biotechnology. Pomona, California

4. Amer, A., **Nolazco, G.M.**, Masri, E., McCaman, R., and Ono, J.K. (2000) Comparisons of nicotinic acetlycholine receptor alpha subunits in muscle and nervous systems of mollusks and annelids. Society for Neuroscience. New Orleans, Louisiana

5. **Nolazco, G.M.,** Nguyen, P., Theisen, H., and McCaman, R. (2001) Gene structure and developmental regulation of the small conductance calcium activated potassium channel gene (dSK) in *Drosophila.* A. *Dros*. Res. Conf. 42 2001: 790A

6. Nguyen, P., **Nolazco, G.M.**, Theisen, H., and McCaman, R. (2001) *Drosophila* K channel gene structure. California State University Program for Education and Research in Biotechnology. Pomona, California

7. Nguyen, P., **Nolazco, G.M.**, Theisen, H., and McCaman, R. (2001) Gene structure and developmental regulation of the small conductance calcium activated potassium channel gene (dSK) in *Drosophila*. 42nd Annual Drosophila Research Conference. Washington, D.C

8. **Nolazco, G.M.**, McCaman, R., and Ono, J.K. (2002) Differential expression of calcium activated potassium channels in *Drosophila*. California State University Program for Education and Researchin Biotechnology. Pomona, California

9. Vernet, D., Magee, T., Qian A., **Nolazco, G**., Rajfer, J., and Gonzalez-Cadavid, NF. (2005) Tadalafil does not upregulate the expression and activity of phosphodiestarase 5 (PDE-5) in the penis. J Urol (1060) 173: 287-288 American Urology Association. San Antonio, Texas

C. Other Support.
None

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Gelfand, Robert Allen	Adjunct Assistant Professor
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Massachusetts Institute of Technology	SB	1970	Mathematics
University of California Santa Barbara	MS	1973	Biological Science
California Institute of Technology	PhD	1980	Biochemistry

A. Positions and Honors
1973 Graduate Fellowship, California Institute of Technology
1980-6 Postdoctoral Fellow, Purdue University Department of Biology
1987-90 Staff Research Associate, University of California Irvine and VA Medical Center Long Beach CA
1990-94 Biochemist, VA Medical Center, Long Beach CA
1995-7 Lecturer, Department of Chemistry and Biochemistry, Cal. State Univ Long Beach
1997-2008 Director, Gelfand Partnership, a management company
2003–7 Columnist of media, culture, and science, American-Reporter.com
2004-8 Editor, Coastal Currents, San Pedro CA
2005-7 Visiting Scientist, LABiomed, Harbor UCLA Medical Center
2007-8 Staff Research Associate IV, LABiomed Harbor UCLA Medical Center
2007-8 Adjunct Assistant Professor, Charles R. Drew University of Medicine and Science
2007-8 Adjunct Faculty, Los Angeles Southwest College

B. Professional Membership

1992-2008 Endocrine Society Member

C. Selected peer-reviewed publications 1990 – 2008 out of a total of 21 peer reviewed papers and chapters.

Note: Due to the loss of grant funding, I left research in 1994. This was compounded by a malignant melanoma in 2000 which was excised and has not returned. In the interim, I developed a business, taught biochemistry and chemistry in the university setting, and published numerous articles in daily newspapers and on the internet, totaling greater than 150 individual pieces on science, culture, and the media (not shown). Since late 2005, I have returned to scientific research and teaching, and I am committed to continuing as long as I am able.

Levin, ER., Frank, HJL., Gelfand, RA., Loughlin, SE., and Kaplan, G. Natriuretic peptide receptors in cultured rat diencephalon. J. Biol. Chem. 265:10019-10024. 1990.

Matsuoka, LY., Wortsman, J., Tang, G., Russell, RM., Parker, L., Gelfand, RA., Mehta, RG. Are endogenous retinoids involved in the pathogenesis of acne? Arch. Derm. 127:1072-3. 1991.

Gelfand, RA., Frank, HJL., Levin, E., Pedram, A. Brain and atrial natriuretic peptides bind to common receptors in brain capillary endothelial cells. Am. J. Physiol. 261:E183-E189. 1991.

Parker, L, Lifrak E, Gelfand, R, Shively J, Lee T, Kaplan B, Walker, P, Calaycay J, Florsheim W, Mason I and Soon-Shiong P. Isolation, purification, synthesis, and binding of human adrenal gland cortical androgen stimulating hormone. End J 1:441-445 (1993).

Frank, H, Pedram, A, Hu, R, Gelfand, R. and Levin, E. Regulation of atrial natriuretic peptide receptors in brain capillary endothelial cells. Endo J. 2:33-40 (1994).

Frank, H, Pedram, A, Hu, R, Gelfand, R. and Levin, E. Regulation of atrial natriuretic peptide receptors in brain capillary endothelial cells. Endo J. 2:33-40 (1994).

Gelfand, R, Wepsic, HT, Parker, L and Jadus, M. Prostaglandin E_2 induces upregulation of murine macrophage b-endorphin receptors. Immunol Lett. 45:143-148 (1995).

Gelfand, R, Bobrow, A, Pham, L, Young, C, and Parker, L. b-endorphin binding in cultured adrenal cortical cells. Endocrine 3:201-207 (1995).

Nolazco G, Kovanecz I, Vernet D, Ferrini M, Gelfand R, Tsao J, Magee T, Rajfer J, Gonzalez-Cadavid NF (2008) Effect of muscle derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. BJU Int, in press

Cantini LP, Ferrini MG, Vernet D, Magee TR, Quian A, Gelfand RA, Rajfer J, Gonzalez-Cadavid NF (2008) Pro-fibrotic role of myostatin in Peyronie's disease. J Sex Med, in press.

c. RESEARCH SUPPORT
None